UNIVERSIDADE ESTADUAL PAULISTA – UNESP

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS (FCAV)

CÂMPUS DE JABOTICABAL

DIVERSIDADE GENÉTICA DE AGENTES TRANSMITIDOS POR VETORES EM QUATIS (*Nasua nasua)* EM ÁREA PERIURBANA NO CENTRO-OESTE BRASILEIRO

Lívia Perles

Médica Veterinária

2023

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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para obtenção do título de Doutora em Medicina Veterinária, área: Patologia Animal.

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Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: DIVERSIDADE GENÉTICA DE AGENTES TRANSMITIDOS POR VETORES EM QUATIS (*Nasua nasua*) EM ÁREA PERIURBANA NO CENTRO-OESTE BRASILEIRO

AUTORA: LIVIA PERLES
ORIENTADOR: MARCOS ROGÉRIO ANDRÉ
COORIENTADORA: ROSANGELA ZACARIAS MACHADO

Aprovada como parte das exigências para obtenção do Título de Doutora em Medicina Veterinária, área: Patologia Animal pela Comissão Examinadora:

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Profa. Dra. JULIA ANGELICA GONÇALVES DA SILVEIRA (Participaçao Virtual)
UFMG / Belo Horizonte/MG

Jaboticabal, 10 de março de 2023

Impacto potencial desta pesquisa

Os quatis (*Nasua nasua*, Carnivora: Procyonidae) estão amplamente distribuídos no Brasil e se adaptam facilmente a ambientes antropizados, tornando possível um contato próximo com animais domésticos e humanos. Diversas bactérias e protozoários são conhecidos pelo seu potencial zoonótico, sendo encontrados em animais e transmitidos aos seres humanos por vetores (e.x. carrapatos, mosquitos, pulgas). Estudar animais silvestres que dividem o ambiente urbano com os seres humanos é importante como uma forma de vigilância de enfermidades emergentes. O presente estudo objetivou investigar a presença e diversidade genética de agentes com potencial zoonótico transmitidos por ectoparasitos em duas áreas urbanas da cidade de Campo Grande, estado do Mato Grosso do Sul. Foi detectada a presença de DNA de agentes bacterianos das famílias Anaplasmataceae e Mycoplasmataceae e de filarídeos em amostras de sangue dos quatis amostrados e DNA de agentes bacterianos das famílias Bartonellaceae e Rickettsiaceae e de protozoários da família Babesiidae nos carrapatos dos animais amostrados. Foi possível observar que os quatis infectados não apresentaram alterações hematológicas e clínicas significativas, sugerindo um possível papel de reservatório desses animais. O potencial zoonótico dos agentes detectados é ainda desconhecido.

Potential impact of this research

Coatis (*Nasua nasua*, Carnivora: Procyonidae) are widely distributed in Brazil that easily adapt to anthropic environments, making close contact with domestic animals and humans possible. Several bacteria and protozoa are known for their zoonotic potential, being found in animals and transmitted to humans by vectors (e.g. ticks, mosquitoes, fleas). Studying wild animals that share the urban environment with humans is important as a form of surveillance of emerging diseases. The present study aimed to investigate the presence and genetic diversity of agents with zoonotic potential transmitted by ectoparasites in two urban areas of the city of Campo Grande, state of Mato Grosso do Sul. The presence of DNA of bacterial agents of the Anaplasmataceae and Mycoplasmataceae families and of filarids was detected in blood samples from the sampled coatis and DNA of bacterial agents of the Bartonellaceae and Rickettsiaceae families and of protozoa of the Babesiidae family in the ticks of the sampled animals. It was possible to observe that the infected coatis did not present significant hematological and clinical alterations, suggesting a possible reservoir role for these animals. The zoonotic potential of the detected agents is still unknown.

DADOS CURRICULARES DA AUTORA

Lívia Perles – Filha de Benedito Aparecido Perles e Roseli Inês Stuqui Perles, nasceu em 26 de fevereiro de 1992, na cidade de Catanduva/SP. Em 2010 ingressou na Universidade Estadual Paulista "Júlio de Mesquita Filho" Câmpus Jaboticabal, onde em janeiro 2015 se formou Médica Veterinária. Realizou durante a graduação duas iniciações científicas sob orientação do Prof. Dr. Maurício Barbanti Duarte no NUPECCE (Núcleo de Pesquisa e Conservação de Cervídeos) com projetos na área de Genética da Conservação, sendo contemplada com duas bolsas da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP #2011/10036-7; 2013/17257-4). No ano de 2015 ingressou no programa de Residência em Área Profissional de Saúde – Medicina Veterinária e Saúde, na subárea Medicina de Animais Selvagens sob orientação da Prof. Dra. Karin Werther. Ingressou no curso de Mestrado do Programa de Pós-graduação em Medicina Veterinária (Patologia Veterinária), em março de 2017, sob orientação do Prof. Dr. Marcos Rogério André, na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista "Júlio de Mesquita Filho" – Campus Jaboticabal, com bolsa de estudo da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Em 2019, ingressou no curso de Doutorado do Programa de Pós-graduação em Medicina Veterinária (Patologia Veterinária) sob orientação do Prof. Dr. Marcos Rogério André e coorientação da Profa. Dra. Rosangela Zacarias Machado, na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista "Júlio de Mesquita Filho" – Câmpus Jaboticabal, inicialmente com bolsa de estudo do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e posteriormente com bolsa FAPESP (#2019/15150-4). Em abril de 2022 recebeu o prêmio "1º Prêmio às Estudantes e Jovens Pesquisadoras da UNESP na Modalidade: II e Área do Conhecimento Ciências Agrárias". Em maio de 2022 iniciou período de pesquisa no exterior com bolsa FAPESP BEPE (2021/14244-5) no Departamento de Medicina Veterinária da Università degli Studi di Bari Aldo Moro, Laboratório de Parasitologia, Bari, Itália, sob supervisão do Prof. Dr. Domenico Otranto.

EPÍGRAFE

“Há que ter alguma coragem.
Há que ter algum sonho correndo nas veias
e um grão de loucura faiscando na alma”.

Lya Luft

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A Deus por nunca me desamparar no meu caminho.

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Aos amigos especiais que fiz na UniBa, Viviane Noll Louzada Flores, Lucas Beltrame, Marcos Bezerra Santos, Andrea Cannarozzi, Jairo Mendoza, Giada Annoscia, Mariaelisa Carbonara, Giovanni Sgroi e Kassim Karama.

SUMÁRIO

DIVERSIDADE GENÉTICA DE AGENTES TRANSMITIDOS POR VETORES EM QUATIS (*Nasua nasua*) EM ÁREA PERIURBANA NO CENTRO-OESTE BRASILEIRO

Resumo - Quatis (*Nasua nasua*) são procionídeos amplamente distribuídos na América do Sul que se adaptam facilmente a ambientes antropizados, vivendo em contato próximo com animais domésticos e humanos. O presente estudo teve como objetivo investigar a diversidade de ectoparasitas e a ocorrência de agentes transmitidos por vetores (Anaplasmataceae, Mycoplasmataceae, Bartonellaceae, Rickettsiaceae Babesiidae/Theileriidae, Hepatozoidae e Onchocercidae) em quatis e/ou ectoparasitas amostrados em duas áreas urbanas de Campo Grande, Mato Grosso do Sul, Brasil. Entre 2018 e 2019, dos 97 quatis capturados/recapturados foram coletados 2.242 carrapatos (838 larvas de *Amblyomma*, com maior infestação em janeiro, abril e maio; 1241 ninfas de *Amblyomma sculptum* e 150 ninfas de *Amblyomma dubitatum*, com maior infestação em julho, agosto, outubro e novembro; 13 adultos, dentre três machos e cinco fêmeas de *A. sculptum*; e dois machos e três fêmeas de *Amblyomma ovale*). Cinquenta e nove piolhos [*Neotrichodectes (Nasuicolla) pallidus*] foram coletados de 25,7% quatis, representando um novo registro da espécie no centro-oeste do Brasil, com a primeira contribuição morfológica dos estágios de ninfa e ovos e caracterização molecular utilizando os genes 18S rRNA e *cox1*. Dos 97 quatis amostrados, 77% mostraram-se positivos para *Hepatozoon procyonis* (gene 18S rRNA); 15,1% para *Ehrlichia* sp. (gene *dsb*); 3,6% para *Anaplasma* sp. (gene 16S rRNA); 89,6% para hemoplasmas (gene 16S rRNA) e 3,6% para *Neorickettsia* sp. Nenhuma amostra de sangue foi positiva para *Bartonella* spp. em cultura ou na qPCR e piroplasmídeos na nPCR (nested PCR). Ao analisar os esfregaços sanguíneos e capa leucocitária, 33% e 80%, respectivamente, das amostras apresentaram pelo menos uma microfilária de *Mansonella* sp. (molecularmente caracterizada com base nos genes *cox1*, *myoHC* and *hsp70*). *Hepatozoon procyonis* foi detectado em alta prevalência em quatis do centro-oeste do Brasil, com parasitemia flutuando entre as recapturas e aparentemente sem influência nos parâmetros hematológicos e clínicos dos quatis parasitados. Análises filogenéticas baseadas no gene 16S rRNA e ITS posicionaram as sequências de *Anaplasma* sp. detectadas no presente estudo em um grande clado com outras sequências *Anaplasma* spp. previamente detectadas em carrapatos e animais silvestres e em um clado com '*Candidatus* Anaplasma brasiliensis', respectivamente. Com base em seis marcadores moleculares distintos, o presente trabalho descreveu um novo agente Anaplasmataceae, nomeado '*Candidatus* Ehrlichia dumleri'. A filogenia baseada nos genes 16S rRNA e 23S rRNA e qPCR baseado no gene 16S rRNA indicaram a presença de dois genótipos de hemoplasmas em quatis. A estimativa da carga bacteriana de hemoplasmas não mostrou associação com indicadores hematológicos de anemia. Embora as amostras positivas do presente estudo tenham apresentado >99% de identidade com *Neorickettsia risticii* disponível no GenBank, a real identidade da bactéria detectada em quatis deve ser melhor investigada por meio de isolamento e sequenciamento do genoma completo. O presente estudo mostrou uma alta prevalência de *Mansonella* sp., sugerindo que quatis podem atuar como hospedeiros de uma nova espécie de *Mansonella* sp. ainda não descrita dentro da família Onchocercidae. Em relação aos carrapatos, 25,4% das amostras de DNA de carrapatos foram positivas para *H. procyonis*, 10% para '*Candidatus* Mycoplasma haematonasua', 1,6% para *Bartonella* spp., 2% para piroplasmídeos [sendo duas sequências diferentes, uma com identidade para *Babesia* sp. previamente detectada em capivaras (*Hydrochoerus hydrochaeris*), e outra com identidade para *Babesia* sp., detectado em gambás (*Didelphis albiventris*) e carrapatos associados *A. dubitatum*] e 0,8% para *Rickettsia* spp. (do Grupo Febre Maculosa [GFM] e *Rickettsia belli*). Nenhum piolho mostrou-se positivo para os agentes acima mencionados. Carnívoros são considerados importante fonte de infecções humanas com agentes zoonóticos transmitidos por vetores. A detecção de *Rickettsia* sp. de SFG destaca a importância de estudos de vigilância em fragmentos florestais urbanos. A detecção de piroplasmas em carrapatos de animais negativos pode indicar a importância de *Amblyomma* spp. na transmissão transovariana/transestadial de *Babesia* spp. em animais silvestres.

Palavras-chave: Ectoparasitas, Rickettsiales, *Bartonella*, hemoplasmas, *Hepatozoon procyonis*, procionídeos, *Mansonella* sp.

GENETIC DIVERSITY OF VECTOR-BORNE AGENTS IN COATIS (*Nasua nasua*) IN A PERIURBAN REGION OF CENTRAL-WESTERN BRAZIL

Abstract – Coatis (*Nasua nasua*) are procyonids widely distributed in South America that easily adapt to anthropized environments, living in close contact with domestic animals and humans. The present study aimed to investigate the diversity of ectoparasites and the occurrence of vector-borne agents (Anaplasmataceae, Mycoplasmataceae, Bartonellaceae, Rickettsiaceae Babesiidae/Theileriidae, Hepatozoidae and Onchocercidae) in coatis and/or ectoparasites sampled in two urban areas of Campo Grande, Mato Grosso do Sul, Brazil. Between 2018 and 2019, 2,242 ticks were collected from 97 coatis captured/recaptured (838 *Amblyomma* larvae, with higher infestation in January, April and May; 1,241 *Amblyomma sculptum* nymphs and 150 *Amblyomma dubitatum* nymphs, with higher infestation in July, August, October and November; 13 adults, among three males and five females of *A. sculptum*; and two males and three females of *Amblyomma ovale*). Fifty-nine lice [*Neotrichodectes (Nasuicolla) pallidus*] were collected from 25.7% coatis, representing a new record of the species in central-western Brazil, with the first morphological contribution to nymph and egg as well as molecular characterization based on the 18S rRNA and *cox1* genes. Out of 97 coatis sampled, 77% were positive for *Hepatozoon procyonis* (18S rRNA gene), 15.1% for *Ehrlichia* sp. (*dsb* gene), 3.6% for *Anaplasma* sp. (16S rRNA gene), 89.6% for hemoplasmas (16S rRNA gene), and 3.6% for *Neorickettsia* sp. No coati blood sample was positive for *Bartonella* spp. in culture or qPCR and piroplasmids in nPCR (nested PCR). When analyzing the coati blood smears and buffy coats, 33% and 80%, respectively, samples showed at least one microfilaria of *Mansonella* sp. (molecularly characterized based on the *cox1, myoHC* and *hsp70* genes). *Hepatozoon procyonis* circulates with high prevalence in coatis in central-west Brazil, with parasitemia fluctuating between recaptures and apparently without influence on the hematological and clinical parameters of the parasitized coatis. Phylogenetic analyzes based on the 16S rRNA gene and ITS positioned the *Anaplasma* sp. detected in the present study in a large clade with other *Anaplasma* spp. previously detected in ticks and wild animals and in a clade with '*Candidatus* Anaplasma brasiliensis', respectively. Based on six distinct molecular markers, the present work described a new Anaplasmataceae agent, named '*Candidatus* Ehrlichia dumleri'. Phylogeny based on 16S rRNA and 23S rRNA genes and qPCR based on 16S rRNA gene indicated the presence of two genotypes of hemoplasmas in coatis. Estimation of the bacterial load of hemoplasmas showed no association with hematological indicators of anemia. Although the positive samples in the present study showed >99% identity with *Neorickettsia risticii* available in GenBank, the real identity of the bacteria detected in coatis should be better investigated through isolation and Whole Genome Sequencing. The present study showed a high prevalence of *Mansonella* sp., suggesting that coatis may act as reservoirs for a new species of *Mansonella* sp. yet to be described. Regarding ticks, 25.4% of tick DNA samples were positive for *H. procyonis*, 10% for '*Candidatus* Mycoplasma haematonasua', 1.6% for *Bartonella* spp., 2% for piroplasmids [among two different sequences: one with identity for *Babesia* sp. previously detected in capybaras (*Hydrochoerus hydrochaeris*), and another one showing for *Babesia* sp. previously detected in opossums (*Didelphis albiventris*) and associated *A. dubitatum* ticks] and 0.8% for *Rickettsia* spp. (from the Spotted Fever Group [SFG] and *Rickettsia bellii*). No louse was positive for the above mentioned agents. Carnivores are considered an important source of human infections with vector-borne zoonotic agents. The detection of SFG *Rickettsia* sp. highlights the importance of surveillance studies in urban forest fragments. The detection of piroplasmids in ticks collected from negative animals may indicate the importance of *Amblyomma* spp. in transovarian/transstadial transmission of *Babesia* spp. in wild animals.

Key-Words: Ectoparasites, Rickettsiales, *Bartonella*, hemoplasmas, *Hepatozoon procyonis*, procyonids, *Mansonella* sp.

UCDB

UNIVERSIDADE CATÓLICA DOM BOSCO
COMISSÃO PARA USO DE ANIMAIS

CERTIFICADO

Certificamos que a proposta intitulada "**O parasitismo por tripanossomatídeos em quatis (Nasua nasua) e capivaras (Hydrochoerus hydrochaeris) em fragmentos florestais urbanos de Campo Grande, MS**", registrada com o nº **001/2018**, sob a responsabilidade de Heitor Miraglia Herrera, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi APROVADA pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) da Universidade Católica Dom Bosco em reunião de 21/09/2018.

Finalidade	Pesquisa Científica
Vigência da autorização	21/09/2018 – 21/09/2020
Espécie/linhagem/raça	Nasua nasua/Hydrochoerus hydrochaeris
Nº de animais	300
Peso/Idade	-
Sexo	-
Origem	Fragmentos florestais da área urbana de Campo Grande, MS

Campo Grande, 21 de setembro de 2018.

Profa. Dra. Susana Elisa Moreno
Presidente da CEUA /UCDB

Profa. Dra. Susana E. Moreno
Presidente do CEUA - UCDB

Comissão para Uso de Animais. Universidade Católica Dom Bosco. Av. Tamandaré 6000. Campo Grande- MS. CEP 79117-900. (67) 3312.3688. ceua@ucdb.br

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Diversidade genética de agentes transmitidos por vetores em Quatis (*Nasua nasua*) em área periurbana no centro-oeste brasileiro"**, protocolo nº 06731/19, sob a responsabilidade do Prof. Dr. Marcos Rogério André, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 13 de junho de 2019.

Vigência do Projeto	01/08/2019 a 01/12/2022
Espécie / Linhagem	Quati (*Nasua nasua*)
Nº de animais	185
Peso / Idade	2 - 8kg/ Jovem e adultos
Sexo	Machos e fêmeas
Origem	Campo Grande – Mato Grosso do Sul

Jaboticabal, 13 de junho de 2019.

Fabiana Pilarski

Prof.ª Dr.ª Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal SP - Brasil
tel 16 3209 7100 www.fcav.unesp.br

Qualidade em Biossegurança para inclusão de áreas com nível de Biossegurança NB1 para execução de atividades com organismos geneticamente modificados da classe de risco 1. As atividades a serem desenvolvidas pela instituição são: Pesquisa em regime de contenção e ensino com animais geneticamente modificados da classe de risco I. Os organismos a serem manipulados são linhagens de camundongos (Mus musculus) nocautes para diversos genes. As instalações a serem credenciadas são denominadas de Biotério da UFCSPA, situ a Rua Sarmento Leite, nº 245, Prédio 3, Sala 415, Centro Histórico, Porto Alegre/RS. O responsável técnico pelas instalações será a Dra. Fernanda Basto de Mello e esta declara que o laboratório dispõe de infraestrutura adequada e pessoal técnico capaz de gerir o risco associado à atividade proposta. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.012/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 214ª Reunião Ordinária da CTNBio, realizada em 01 de agosto de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº: 01250.024938/2018-73

Requerente: Universidade Federal do Rio Grande do Sul (UFRGS)

CQB: 060/98

Endereço: Av. Bento Gonçalves, nº 9.500, Prédio 43.431 - Campus do Vale/UFRGS C.P.15.005 - CEP 91.501-970. Porto Alegre-RS.

Assunto: Solicitação de extensão de CQB e atualização das informações do Laboratório de Radiobiologia Molecular do Centro de Biotecnologia no CQB da instituição.

Extrato Prévio: nº 6116/17, publicado no DOU em 19 de julho do 2018.

Decisão: Deferido

A CTNBio, após apreciação da Solicitação de parecer técnico para extensão de Certificado de Qualidade em Biossegurança para atividades de pesquisa em regime de contenção com OGM da classe de risco I em instalações com nível de biossegurança NB-1, concluiu pelo deferimento, nos termos deste Parecer Técnico. O Presidente do Comissão Interna de Biossegurança da Universidade Federal do Rio Grande do Sul - UFRGS, Dr. Giancalo Pasquali, solicita à CTNBio parecer técnico para extensão do Certificado de Qualidade em Biossegurança (CQB) da instituição para atualização das informações do Laboratório de Radiobiologia Molecular do Centro de Bitecnologia (CBiot) da UFRGS. As instalações tiveram o nome modificado para Grupo de Estudos de Leveduras Cervejeiras (GELO) e estará sob a responsabilidade do Dr. Diego Bonatto e localizado no endereço: Centro de Biotecnologia - Lab. 107 - Av. Bento Gonçalves, 9.500, Prédio 43.421, Campus do Vale/UFRGS - Porto Alegre, RS. C.P. 15.005, CEP 91.501-970. As atividades a serem desenvolvidas nas instalações são: Pesquisa em regime de contenção e ensino com organismos geneticamente modificados da classe de risco 1. Os organismos a serem manuseados pela instituição nestas instalações são linhagens de comerciais da bactéria Escherichia coli derivadas de K12 e leveduras Saccharomyces cerevisiae, linhagens laboratoriais derivadas de BY e cepas industriais da classe de risco 1. O responsável pela unidade operativa declara que as instalações contam com salas e equipamentos úteis em nível de biossegurança adequado às atividades propostas. O processo descreve as condições de biossegurança das áreas a serem cadastradas, as medidas de biossegurança propostas para o laboratório e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização dos projetos propostos. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.013/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 214ª Reunião Ordinária da CTNBio, realizada em 01 de agosto de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº: 01250.026216/2018-53

Requerente: Instituto Oswaldo Cruz - Fiocruz

CQB: 105/99

Endereço: Av. Brasil, 4365. Pav. Gomes de Farias Sala 210, Manguinhos - Rio de Janeiro - RJ - CEP 21045-900.

Assunto: Solicitação de extensão de CQB para instalações para execução de atividades com OGMs da classe 1 de risco biológico.

Extrato Prévio: nº 6115/18, publicado no DOU em 21 de maio do 2018.

Decisão: Deferido

A CTNBio, após apreciação da Solicitação de parecer técnico para extensão de Certificado de Qualidade em Biossegurança para atividades de pesquisa em regime de contenção com OGM da classe de risco I em instalações com nível de biossegurança NB-1, concluiu pelo deferimento, nos termos deste Parecer Técnico.O Presidente do Comissão Interna de Biossegurança do Instituto Oswaldo Cruz, Dr. Ricardo Cunha Machado, solicita à CTNBio parecer técnico para extensão do Certificado de Qualidade em Biossegurança (CQB) da instituição para inclusão da área do Laboratório de Comunicação Celular (LCC) do Instituto Oswaldo Cruz para execução de atividaddes de pesquisa em regime de contenção com organismos geneticamente modificados da classe de risco biológico 1 nas instalações da instituição. As instalações a serem credenciadas são denominadas: Laboratório de Comunicação Celular (LCC), situado no Pavilhão 108, Sala 33 e 38B- Manguinhos - Av. Brasil, 4365, Rio de Janeiro. CEP: 21040360. A instituição solicita que as áreas sejam credenciadas para o nível de biossegurança 1 junto a CTNBio. Os organismos a serem manuseados pela instituição nestas instalações são linhagens de comerciais da bactéira Escherichia coli contendo genes de Homo sapiens da classe de risco 1. O projeto a ser desenvolvido denomina-se: "Receptor P2X7: análise estrutural e funcional a possíveis aplicações clínicas", sob a responsabilidade do Dr. Luiz Anastacio Alves. O responsável pela unidade operativa declara que as instalações contam com salas e equipamentos úteis em nível de biossegurança adequado às atividades propostas. O processo descreve as condições de biossegurança das áreas a serem cadastradas, as medidas de biossegurança propostas para o laboratório e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização dos projetos propostos. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.014/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 214ª Reunião Ordinária da CTNBio, realizada em 01 de agosto de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº: 01250.026233/2018-91

Requerente: Faculdade de Ciências Agrárias e Veterinárias - Universidade Estadual Paulista, UNESP, Câmpus de Jaboticabal.

CQB: 088/98

Endereço: Via de Acesso Prof. Paulo Donato Castellane, s/n. CEP 14884-900 - Jaboticabal - SP.

Assunto: Solicitação de extensão de CQB para instalações para execução de atividades com OGMs da classe 1 de risco biológico.

Extrato Prévio: nº 6115/18, publicado no DOU em 21 de maio do 2018.

Decisão: Deferido

A CTNBio, após apreciação da Solicitação de parecer técnico para extensão de Certificado de Qualidade em Biossegurança para atividades de pesquisa em regime de contenção com OGM da classe de risco I em instalações com nível de biossegurança NB-1, concluiu pelo deferimento, nos termos deste Parecer Técnico.O Presidente do Comissão Interna de Biossegurança da Faculdade de Ciências Agrárias e Veterinárias - Universidade Estadual Paulista, UNESP, Câmpus de Jaboticabal, Dr. Odair Aparecido Fernandes, solicita à CTNBio parecer técnico para extensão do Certificado de Qualidade em Biossegurança (CQB) da instituição para inclusão do Laboratório de Imunoparasitologia do Departamento de Patologia Veterinária para execução de atividades de pesquisa em regime de contenção, descarte, ensino e armazenamento com organismos geneticamente modificados da classe de risco biológico 1 nas instalações da instituição. As instalações a serem credenciadas são denominadas: Laboratório de Imunoparasitologia do Departamento de Patologia Veterinária, situado na Via de Acesso Prof. Paulo Donato Castellane, s/n. CEP 14884-900, Jaboticabal - SP. A instituição solicita que as áreas sejam credenciadas para o nível de biossegurança 1 junto a CTNBio. Os organismos a serem manuseados pela instituição nestas instalações são linhagens de comerciais da bactéria Escherichia coli contendo fragmentos dos genes codificadores de antígeno de superfície de merozoíto msa-1, msa- 2b e msa- 2c do protozoário Babesia bovis da classe de risco 1. O projeto a ser desenvolvido denomina-se: "Diversidade genética de Babesia bovis em bovinos de corte amostrados no pantanal sul matogrossense", sob a responsabilidade do Dr. Marcos Rogério André. O responsável pela unidade operativa declara que as instalações contam com salas e equipamentos úteis em nível de biossegurança adequado às atividades propostas. O processo descreve as condições de biossegurança das áreas a serem cadastradas, as medidas de biossegurança propostas para o laboratório e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização dos projetos propostos. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.017/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 214ª Reunião Ordinária da CTNBio, realizada em 01 de agosto de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº 01250.010280/2018-12

Requerente: Instituto Oswaldo Cruz.

CQB: 105/99

Endereço: Instituto Oswaldo Cruz. Av. Brasil, 4365 - Pav. Gomes de Farias - Sala 114. Manguinhos. Rio de Janeiro, RJ - CEP 21045-900. Tel. 21-2598-4440 - Fax: 21-2560-7864.

Assunto: Solicitação de parecer para execução de projeto com organismo geneticamente modificado da classe de risco 2.

Extrato Prévio: nº 5992/17, publicado no DOU em 21 de maio do 2018.

Decisão: Deferido

A CTNBio, após apreciação da Solicitação de parecer técnico para execução de projeto com organismo geneticamente modificado da classe de risco 2 em instalações com nível de biossegurança NB-2, concluiu pelo deferimento, nos termos deste Parecer Técnico. O presidente da Comissão Interna de Biossegurança da Fundação Oswaldo Cruz, Dr. Ricardo Cunha Machado, solicita à CTNBio parecer técnico para execução de projeto com organismo geneticamente modificado da classe de risco 2. As instalações nas quais será conduzido o projeto são as do Laboratório de Imunologia Clínica do Instituto Oswaldo Cruz, situado a Pavilhão Leônidas Deane, 4º andar, sala 409-C, Av. Brasil, 4365- Manguinhos- Rio de Janeiro - RJ, CEP. 21045-900. O projeto a ser executado é denominado "Avaliação in vitro do Delta BCG Moreau e Pasteur no Brasil", será coordenado pelo Dr. Paulo Renato Zuquim Antas, em nível de contenção NB-2. Os organismos a serem manipulados são Cepas recombinantes de Mycobacterium bovis Bacilo Calmette-Guerin (BCG) que já foram desprovidas dos genes BCG1419c (denominado BCGABCG1419c), e BCG1416c (denominado BCGABCG1416c). O responsável pela unidade operativa declara que o laboratório conta com equipamentos úteis para as atividades experimentais em nível de biossegurança adequado. O processo descreve as condições de biossegurança das áreas a serem cadastradas, as medidas de biossegurança propostas para o laboratório e a qualificação da equipe de pesquisadores envolvida no projeto, bem como a declaração formal do responsável assegurando que as condições descritas no processo são apropriadas à realização dos projetos propostos. No âmbito das competências dispostas na Lei 11.105/05 e seu decreto 5.591/05, a Comissão concluiu que o presente pedido atende às normas da CTNBio e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

MARIA SUELI SOARES FELIPE

EXTRATO DE PARECER TÉCNICO Nº 6.018/2018

A Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 210ª Reunião Ordinária da CTNBio, realizada em 08 de março de 2018, a Comissão apreciou e emitiu parecer técnico para o seguinte processo:

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO
SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº ABDF0B5

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**ABDF0B5**
Usuário:	**Marcos Rogério André**
CPF/CNPJ:	**302.435.148-59**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Nasua nasua
Amblyomma dubitatum
Amblyomma sculptum
Amblyomma ovale
Neotrichodectes pallidus
Nasua nasua
Amblyomma dubitatum
Amblyomma sculptum
Amblyomma ovale
Neotrichodectes pallidus

Título da Atividade:	**DIVERSIDADE GENÉTICA DE AGENTES TRANSMITIDOS POR VETORES EM QUATIS (Nasua nasua) EM ÁREA PERIURBANA NO CENTRO-OESTE BRASILEIRO**

Equipe

Marcos Rogério André	**Campus de Jaboticabal**
Livia Perles	**Unesp FCAV**
Rosangela Zacarias Machado	**Unesp FCAV**
Heitor Miraglia Herrera	**UCDB**

Parceiras Nacionais

03.226.149/0001-81 / Universidade Católica dom Bosco

Parceiras no Exterior

University of Bari Aldo Moro

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto intitulado **"Carnívoros selvagens generalistas como sentinelas para filarioses e leishmaniose visceral no Parque Nacional do Iguaçu"**, protocolo nº 11.510/16, sob a responsabilidade do Prof. Dr. Estevam Guilherme Lux Hoppe, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 05 de outubro de 2016.

Vigência do Projeto	06/10/2016 a 01/08/2020
Espécie / Linhagem	Quatis (*Nasua nasua*), graxains (*Cerdocyon thous*), outros carnívoros
Nº de animais	Estima-se em 80 animais
Peso / Idade	Variados
Sexo	Machos e fêmeas
Origem	Parque Nacional do Iguaçu

Jaboticabal, 05 de outubro de 2016.

Profª Drª Lizandra Amoroso
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n - 14884-900 - Jaboticabal - SP - Brasil
tel 16 3209 2600 fax 3202 4275 www.fcav.unesp.br

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Comprovante de Cadastro de Acesso

Cadastro nº AAA5680

A atividade de acesso ao Patrimônio Genético, nos termos abaixo resumida, foi cadastrada no SisGen, em atendimento ao previsto na Lei nº 13.123/2015 e seus regulamentos.

Número do cadastro:	**AAA5680**
Usuário:	**Marcos Rogério André**
CPF/CNPJ:	**302.435.148-59**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Nasua nasua

Título da Atividade:	**Co-infecção por múltiplos agentes vetoriais em quatis de cauda anelada de vida livre Nasua nasua (Carnivora: Procyonidae) no Parque Nacional do Iguaçu, sul do Brasil, com a primeira evidência molecular de infecção por Bartonella machadoae em Procyonids**

Equipe

Marcos Rogério André	**Campus de Jaboticabal**
Estevam Guilherme Lux Hoppe	**Unesp FCAV**

Data do Cadastro:	**25/10/2022 22:22:57**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **22:23** de **25/10/2022**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

Capítulo 1 – Considerações gerais

1. Família Procyonidae

A família Procyonidae representa uma família de animais da ordem Carnivora, englobando 13 espécies que ocorrem nas Américas, tais como os guaxinins, quatis, mão-peladas, juparás e olingos (Koepfli et al., 2007). No Brasil, são registradas quatro espécies: Jupará-verdadeiro (*Potus flavus*), Mão-pelada (*Procyon cancrivorus*), Olingo-jupará (*Bassaricyon gabbii*) e Quati (*Nasua nasua*) (Gompper, Decker 1998). Os quatis (*N. nasua* Linnaeus, 1766) estão distribuídos amplamente na América do Sul, ocupando áreas desde a Colômbia e Venezuela ao norte até Uruguai e Argentina ao sul (Gompper, Decker 1998) (**Figura 1**). No Brasil são encontrados em todos os biomas e, nos locais onde estão presentes, geralmente são os carnívoros mais abundantes (Robinson, Redford 1986; Emmons, Helgen 2016).

Figura 1. Distribuição geográfica de *Nasua nasua*. Fonte: IUCN (*International Union for Conservation of Nature*) 2008. *Nasua nasua*. *The IUCN Red List of Threatened Species*. Version 2022-1.

Os quatis possuem uma coloração cinzento-amarelada, sendo as regiões ventral e lateral do corpo mais claras, com o focinho preto e alongado. As orelhas são pequenas e arredondadas, com pelos esbranquiçados, que também podem estar presentes na face. Os pés e as mãos são pretos, e a cauda é longa e peluda, medindo entre 22-70 centímetros, apresentando anéis negros característicos da espécie. Possuem uma altura média de 30 centímetros e comprimento corpóreo entre 40-66 centímetros, apresentando em média, 4 quilos de massa corporal, podendo atingir até 11 quilos. São mamíferos com hábitos diurnos e, normalmente, dormem e fazem ninhos em árvores para proteger os filhotes (Hirsch 2007; Olifiers et al., 2009). Estudos prévios sobre a espécie a descrevia como solitária, com machos adultos vivendo separadamente do grupo, enquanto fêmeas e jovens imaturos formavam grupos de até 30 indivíduos, semelhantemente ao descrito para a espécie *Nasua narica*. Fêmeas e machos adultos compartilhariam o território somente durante a época de acasalamento (Crespo 1982; Emmons, Feer 1990). Entretanto, estudos recentes sobre padrões alimentares e comportamento de *N. nasua* mostraram que os hábitos comportamentais da espécie são diferentes quando comparados àqueles de *N. narica*, com a observação de machos adultos convivendo com fêmeas e filhotes fora da época de acasalamento em cinco populações distintas no Brasil (Parque das Mangabeiras - Minas Gerais; Parque Ecológico do Tietê - São Paulo; Fazenda Nhumirim – Mato do Sul; Parque Estadual do Prosa - Mato Grosso do Sul; e Ilha do Campeche - Santa Catarina) (Alves-Costa et al., 2004; Resende et al., 2004; Bonatti 2006; Costa, Mauro 2008; Estevam et al., 2020; Barreto et al., 2021).

A espécie possui hábito alimentar generalista, com uma dieta bastante diversificada, composta de frutas, invertebrados, pequenos vertebrados e alguns vegetais, podendo atuar na dispersão de sementes e regeneração florestal (Alves-Costa et al., 2004; Beisiegel, Mantovani 2006; Alves-Costa, Eterovick 2007; Hirsch 2011a, b). Por possuírem dieta diversificada e serem animais com alta plasticidade, o contato próximo com animais

domésticos e humanos torna-se frequente, já que a espécie se adapta muito bem a áreas peri-urbanas (Rodrigues et al., 2006). Levando em consideração que os quatis estão presentes em todas as regiões do Brasil e em diversas áreas com estreito contato com seres humanos e animais domésticos, é importante a realização de estudos a fim de investigar o possível papel que os mesmos desempenham como fontes de infecção de agentes zoonóticos emergentes.

2. Ectoparasitas em Procionídeos

As zoonoses veiculadas por vetores constituem cerca de 22,8% das doenças emergentes (Guimarães et al., 2001). O contato constante entre animais silvestres, animais domésticos e humanos constitui fator de risco para a (re)-emergência de doenças transmitidas por vetores. Tendo em vista que o contato entre a população humana e animais domésticos e silvestres vem crescendo devido a alterações ambientais, ectoparasitas podem intermediar essa conexão, podendo atuar como vetores de diversos agentes (e.x. protozoários, bactérias, vírus). Compreender a diversidade e ecologia dos ectoparasitas em animais silvestres é essencial para a vigilância de doenças transmitidas por vetores (Ramos et al., 2020).

No que diz respeito à ocorrência de ectoparasitas em quatis no Brasil, diversos estudos foram realizados para compreender a diversidade de artrópodes parasitando a espécie. *Amblyomma brasiliense* e *Amblyomma ovale* foram descritas em *N. nasua* capturados no Paraná; *A. ovale*, larvas de *Amblyomma* sp., *Rhipicephalus (Boophilus) microplus*, *Rhipicephalus sanguineus*, e *Amblyomma cajennense* em Mato Grosso, São Paulo e Minas Gerais; *Amblyomma rotundatum* no Pará, e larvas de *Amblyomma* sp. em Goiás (Barros, Baggio, 1992; Figueiredo et al., 1999; Labruna et al., 2005; Rodrigues et at., 2006; Maia, 2012, Dantas-Torres et al., 2010). Em estudo realizado no Pantanal do estado do Mato Grosso do Sul, quatis foram encontrados infestados por *Amblyomma sculptum*, *Amblyomma*

parvum, *A. ovale*, *R. microplus* e larvas de *Amblyomma* spp. (Sousa et al., 2017a, b, c, d). Recentemente, carrapatos das espécies *Haemaphysalis juxtakochi, A. brasiliense, Amblyomma coelebs* e *A. ovale foram* coletados de quatis no Parque Nacional do Iguaçu, estado do Paraná (Magalhães-Matos et al., 2022). Além de carrapatos, quatis foram encontrados parasitados por piolhos *Neotrichodectes (Nasuicola) pallidus* e por pulgas *Ctenocephalides felis felis e Rhopalopsyllus lutzi lutzi* em Juiz de Fora e no Parque das Mangabeiras, estado de Minas Gerais (Rodrigues et al., 2006; Estevam et al., 2020). No estado de São Paulo, nos municípios de Botucatu e Palmital, infestações por *Amblyomma dubitatum*, *A. ovale, A. sculptum, R. sanguineus* e pulgas *C. felis felis* e *R. lutzi lutzi* foram relatadas em quatis (Silva et al., 2018).

3. Hemoparasitas em Procionídeos

3.1 *Hepatozoon procyonis*

O gênero *Hepatozoon* compreende protozoários hemoparasitas do filo Apicomplexa que infectam uma ampla variedade de hospedeiros vertebrados em todo o mundo (Smith, 1996; Modrý et al., 2017). O ciclo de vida de *Hepatozoo*n spp. inclui um hospedeiro definitivo invertebrado (Smith, 1996), o qual pode ser representado por carrapatos, pulgas, moscas, mosquitos ou piolhos, e um hospedeiro intermediário vertebrado, o qual pode ser um mamífero, réptil, ave ou anfíbio (Lainson et al., 2003; Vincent-Johnson, 2003; Watkins et al., 2006; Rubini et al., 2009). Diferentemente des outras bactérias e protozooários transmitidas por carrapatos, *Hepatozoon* spp.é transmitido quando o hospedeiro intermediário ingere o hospedeiro definitivo, um invertebrado contendo oocistos de *Hepatozoon* (Baneth et al., 2003). A infecção também pode ser adquirida por transmissão intrauterina ou predação de um outro vertebrado infectado, contendo cistos de *Hepatozoon* em órgãos (Maia et al., 2014; Modrý et al., 2017; Hodžić et al., 2018).

Diversas espécies de carrapatos atuam como vetores de infecções por *Hepatozoon* para os vertebrados. O vetor conhecido de *Hepatozoon americanum* é o carrapato *Amblyomma maculatum* (Ewing et al., 2002; Vincent-Johnson, 2003), que é uma espécie de artrópode que já foi relatada em países da América Central e do Norte (Guglielmone et al., 2003; Teel et al., 2010). Nas regiões tropicais e subtropicais do mundo todo, incluindo Estados Unidos, sabe-se que *Hepatozoon canis* é transmitido pelos carrapatos *A. ovale* (Forlano et al., 2005; Forlano et al., 2007; Rubini et al., 2009), *Haemaphysalis longicornis, Haemaphysalis flava* (Murata et al., 1995), e *R. sanguineus* sensu lato (Baneth et al., 2001; Gianelli et al., 2013). Em Minas Gerais, oocistos esporulados de *H. canis* foram observados em *R. microplus* coletados de cães domésticos naturalmente infectados (Miranda et al., 2011). Na região do Pantanal matogrossense, DNA de *H. canis* foi detectado pela PCR em carrapatos *A. sculptum* coletados de cães domésticos naturalmente infectados (Melo et al., 2016). Os autores relataram que possivelmente o DNA detectado poderia ter origem no sangue ingerido pelo artrópode, uma vez que *A. sculptum* não parece ter importância na transmissão de *Hepatozoon* spp. no Brasil (Demoner et al., 2013).

Poucos estudos foram realizados até o presente momento em relação à infecção por *Hepatozoon* spp. em animais da família Procyonidae. A primeira detecção de *Hepatozoon* neste grupo de animais foi realizada nos Estados Unidos em guaxinins (*Procyon lotor)* e, com base nas características morfológicas e morfométricas dos gamontes observados nos esfregaços sanguíneos, a espécie foi nomeada *Hepatozoon procyonis* (Richards, 1961). No Texas, EUA, foram avaliadas lâminas histopatológicas de fragmentos de coração, baço e musculatura esquelética de 65 guaxinins (*Procyon lotor)* em busca de lesões histológicas compatíveis com infecção por *Hepatozoon* spp. Dos animais amostrados, 56/65 (88%) apresentavam gametócitos de *Hepatozoon* sp. em granulomas no miocárdio, 44/65 (68%) esquizontes na musculatura cardíaca e 24/65 (37%) esquizontes na musculatura esquelética; em dois animais (3,07%) foram encontrados esquizontes nas trabéculas esplênicas (Clark et al., 1973).

No Brasil, *H. procyonis* foi diagnosticado pela primeira vez em *N. nasua* no Rio de Janeiro, por análise morfológica de gamontes em esfregaços sanguíneos (Rodrigues et al., 2007). Sousa e colaboradores (2017c) detectaram, pela primeira vez, DNA de *Hepatozoon* spp. por meio de PCR baseada no gene 18S rRNA em quatis no Brasil. No Pantanal do Mato Grosso do Sul (MS), 13/31 (41,9%) dos quatis amostrados mostraram-se positivos na PCR para o referido agente. As sequências 18S rRNA obtidas apresentaram 98-99% de identidade com *Hepatozoon felis* (número de acesso GenBank: KM435071, AB771501). As análises filogenéticas agruparam as sequências detectadas em quatis em um grande clado com sequências de *H. felis*, *H. canis* e *H. americanum* (Sousa et al., 2017c). Em quatis amostrados em Botucatu, no estado de São Paulo, foram encontrados gametócitos morfologicamente semelhantes aos de *H. procyonis* em esfregaços sanguíneos de 9/83 (10,8%) animais. DNA de *Hepatozoon* foi detectado em 21/83 (25,3%) quatis. Com base nas características morfológicas e morfométricas dos gamontes, conclui-se que os animais estavam infectados por *H. procyonis* (Silva et al., 2018). As análises filogenéticas agruparam as sequências detectadas nos quatis em um clado com sequências de *Hepatozoon* sp. detectados em quatis no Pantanal por Sousa e colaboradores (2017c). Recentemente, Estevam et al. (2020) monitoraram uma população de quatis no estado de Minas Gerais durante sete anos. Foram realizados exames moleculares (PCR), parasitológicos (esfregaços de sangue) e avaliação clínica (condição corporal, coloração de mucosas e condição da pelagem). Apesar de os autores observarem que 13% (20/151) dos quatis apresentavam algumas alterações clínicas, como pelagem eriçada, mucosa pálida, cicatrizes e linfonodos ingurgitados, não houve associação entre infecção por *H. procyonis* e estado de saúde. Embora tenha sido demonstrado que *H. procyonis* ocorre em quatis de diferentes estados do Brasil (Rodrigues et al., 2006; da Silva et al., 2018, Sousa et al., 2017c; Estevam et al., 2020), pouco se sabe sobre sua dinâmica de infecção em animais naturalmente infectados e as consequências para o hospedeiro. Portanto, são necessários

estudos objetivando o acompanhamento de populações de quatis naturalmente infectadas com *H. procyonis*, para avaliação do impacto do parasita na saúde dos animais.

3.2 Agentes Anaplasmataceae

A família Anaplasmataceae pertencente à ordem Rickettsiales e é composta pelos gêneros *Ehrlichia, Anaplasma, Neoehrlichia, Neorickettsia* e *Wolbachia*. Engloba alfa-proteobactérias Gram-negativas intracelulares obrigatórias que infectam principalmente neutrófilos, monócitos, macrófagos, plaquetas e células endoteliais de mamíferos, formando vacúolos intracitoplasmáticos conhecidos como mórulas (Dumler et al., 2001; Ismael et al., 2010, 2017; André et al., 2018; Rar et al., 2021). Após a reclassificação dos membros desta família por Dumler e colaboradores em 2001, atualmente o gênero *Ehrlichia* é composto pelas espécies *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia muris, Ehrlichia ruminantium* e *Ehrlichia minasensis*; o gênero *Anaplasma*, por sua vez, é composto por *Anaplasma phagocytophilum, Anaplasma marginale, Anaplasma centrale, Anaplasma ovis, Anaplasma bovis, Anaplasma platys, Anaplasma odocoilei* e *Anaplasma capra; Neorickettsia* por *Neorickettsia sennetsu*, *Neorickettsia helminthoeca*, *Neorickettsia risticii* e *Neorickettsia findlayensis* sp. nov.; *Neoehrlichia* por *Neoehrlichia mikurensis, Neoehrlichia lotoris*, e *Neoehrlichia chilensis*; por fim, o gênero *Wolbachia* possui somente a espécie *Wolbachia pipientis*, que é dividida em diversos supergrupos (Dumler et al., 2001; Atif, 2006; Yabsley et al., 2008b; André et al., 2018; Muller et al., 2018; Portillo et al., 2018; Teymournejad et al., 2020; Patterson et al., 2021; Manoj et al., 2021). Além das espécies já descritas, diversos '*Candidatus*' foram propostos atualmente com auxílio de métodos moleculares, tais como 'C*andidatus* Ehrlichia walkerii', '*Candidatus* Ehrlichia occidentalis', '*Candidatus* Ehrlichia khabarensis', '*Candidatus* Ehrlichia ornithorhynchid', '*Candidatus* Ehrlichia hydrochoerus' (Brouqui et al., 2003; Gofton et al., 2016, 2017, 2018; Rar et al., 2015; Dahmani et al., 2017a; Vieira et al., 2022). Da mesma forma, em relação ao gênero

Anaplasma, '*Candidatus* Anaplasma boleense'; '*Candidatus* Anaplasma mediterraneum', '*Candidatus* Anaplasma corsicanum', '*Candidatus* Anaplasma dongdaense', '*Candidatus* Anaplasma sphenisci', '*Candidatus* Anaplasma brasiliensis', '*Candidatus* Anaplasma amazonensis', '*Candidatus* Anaplasma sparouinense' foram propostos (Kang et al., 2014; Dahmani et al., 2017b; Guo et al., 2018; Vanstreels et al., 2018; Dahmani et al., 2019; Calchi et al., 2020; Duron et al., 2022).

Agentes dos gêneros *Ehrlichia* e *Anaplasma* são transmitidos por vetores artrópodes. Vinte espécies de carrapatos estão envolvidas na transmissão de *A. marginale* e *A. phagocytophilum*, sendo os principais gêneros *Ixodes* e *Haemaphysalis* (para *A. phagocytophilum*), *Dermacentor* (para *A. phagocytophilum* e *A. marginale*), *Rhipicephalus* e *Hyalomma* (para *A. marginale*). As espécies *A. centrale* e *A. platys* são transmitidas principalmente por *Rhipicephalus* spp.; *A. bovis* por *Haemaphysalis* spp. e, menos frequentemente, por *Dermacentor* spp. e *Riphicephalus* spp.; e *A. ovis* pode ser transmitido por *Dermacentor* spp., *Haemaphysalis* spp., *Hyalomma* spp., *Ixodes* spp. e *Rhipicephalus* spp. (Silaghi et al., 2017; Rar et al., 2021). Em cães, a transmissão de *E. canis* ocorre pela linhagem tropical de *R. sanguineus* sensu lato (Moraes-Filho et al., 2015). Carrapatos *Amblyomma* spp. estão relacionados à transmissão de *E. ewingii, E. chaffeensis* e *E. ruminantium*; *Dermacentor* e *Ixodes* também podem ter um papel na transmissão de *E. chaffeensis; E. muris* é transmitido principalmente por *Ixodes* spp. e *Haemaphysalis* spp. carrapatos (Ismail et al., 2010; Rochlin, Toledo, 2020); *E. minasensis* foi isolado de *R. microplus* e bovinos naturalmente infectados (Cabezas-Cruz et al., 2016); *N. mikurensis* é transmitido na Europa pelo carrapato *Ixodes ricinus* (Portillo et al., 2018).Diferentemente dos outros gêneros da família Anaplasmataceae, a infecção por *Neorickettsia risticii* é normalmente adquirida pela ingestão acidental de insetos ou caramujos contendo trematódeos encistados infectados com *N. risticii* (Gibson et al., 2005; Greiman et al., 2013). A bactéria é liberada no lúmen do trato gastrointestinal, invade e se multiplica nas células epiteliais do cólon, posteriormente se transloca para o sangue e infecta monócitos, mastócitos e macrófagos (Lin et al., 2009).

Diversas espécies da família Anaplasmataceae são responsáveis por zoonoses de grande importância. Já foram descritas infecções humanas por *E. chaffeensis* (Erliquiose

Monocítica Humana), *E. ewingii* (Erliquiose Granulocítica), *A. phagocytophilum* (Anaplasmose Granulocítica), *Ehrlichia muris* subsp. *euclariensis*, *A. platys*; *N. mikurensis* e *N. sennetsu* (Paddok, Childs 2003; Thomas et al., 2009; Welinder-Olsson et al., 2010; Arraga-Alvarado et al., 2014; Maggi et al., 2014; Breitschwerdt et al., 2014; Dittrich et al., 2015; Karbowiak et al., 2016; Pritt et al., 2017; McQueen, Centellas 2022). Os sinais clínicos gerados pela infecção por membros deste grupo de bactérias são semelhantes e são caracterizados por febre, dores de cabeça, mialgia, erupções cutâneas, dor abdominal, vômito, diarreia, sinais neurológicos e falência de órgãos em casos mais graves (Doudier et al., 2010; Ismail et al., 2010). No Brasil, evidência sorológica de exposição à *E. chaffeensis* foi relatada em dois indivíduos que apresentavam sintomatologia sugestiva de erliquiose no estado de Minas Gerais (Calic et al., 2004). Estudos demonstraram a presença de anticorpos anti-*E. chaffeensis* em pessoas assintomáticas nos estados de Minas Gerais e Paraná (Costa et al., 2006; Vieira, et al., 2013). Além de relatos de infecção por agentes conhecidos por causar doenças em humanos, detecção molecular de *A. platys* foi relatada em humanos na Venezuela (Arraga-Alvarado et al., 2014 e Estados Unidos (Maggi et al., 2014; Breitschwerdt et al., 2014), sugerindo um potencial zoonótico desse agente, e demonstrando a importância da vigilância dos agentes Anaplasmataceae. Na Venezuela, 20 pessoas apresentaram sinais clínicos compatíveis com Erliquiose Monocítica Humana, sendo que 30% (6/20) apresentaram amostras de sangue positivas na PCR para *E. canis* baseada no gene 16S rRNA (Perez et al., 2006). Adicionalmente, em um banco de sangue humano em Costa Rica, foram encontrados anticorpos anti-*E. canis* em 35% (35/100) das amostras analisadas. Nesse mesmo estudo, foram detectadas 10/280 (3,6%) amostras de sangue positivas para *Ehrlichia* spp. na PCR. O sequenciamento das amostras baseado nos genes *dsb* e *trp36* revelaram um novo genótipo de *E. canis* nas amostras analisadas (Bouza-Mora et al., 2017).

Diversos estudos relataram a presença de agentes da família Anaplasmataceae em animais selvagens no Brasil. Um novo genótipo de *Ehrlichia* spp. foi detectado em felídeos

selvagens mantidos em cativeiro em zoológicos do estado de São Paulo e Brasília (André et al., 2010). Enquanto a filogenia pelo gene 16S rRNA mostrou proximidade dos genótipos encontrados com sequências de *E. canis*, a inferência filogenética pelo gene *omp-1* posicionou-os em um clado distinto, entre sequências de *Ehrlichia* spp. e *Anaplasma* spp., sugerindo a circulação de uma nova espécie de *Ehrlichia* sp. neste grupo de mamíferos (André et al., 2010). Genótipos de *Ehrlichia* spp. filogeneticamente relacionados a *E. ruminantium* foram identificados em onças-pintadas (*Panthera onca*) no Pantanal Sul-Matogrossense (Widmer et al., 2011) e em amostras de baço, pulmão e sangue de cachorros-do-mato (*Cerdocyon thous*) no Espírito Santo (Almeida et al., 2013). André e colaboradores (2012) detectaram agentes da família Anaplasmataceae em carnívoros selvagens mantidos em cativeiro. Amostras de sangue de 10/100 (10%) de canídeos (1 lobo europeu [*Canis lupus lupus*], 3 cachorros vinagre [*Speothos venaticus*] e 6 cachorros do mato) e de 21/99 (21%) felídeos (4 pumas [*Puma concolor*], 6 gatos do mato pequeno [*Leopardus tigrinus*], 4 jaguatiricas [*Leopardus pardalis*], 3 gatos mourisco [*Herpailurus yagouaroundi]*, 1 tigre [*Panthera tigres*] e 3 leões [*Panthera leo*]) mostraram-se positivos na PCR para o gene 16S rRNA de *Ehrlichia* spp. Análises filogenéticas agruparam as sequências obtidas em um clado relacionado a *E. chaffeensis* e *E. canis* (André et al., 2012). No Pantanal sul-matogrossense, genótipos 16S rRNA filogeneticamente próximos a *E. canis* e às sequências de *Ehrlichia* spp. obtidas de gansos-do-Orinoco (*Neochen jubata*) e felinos selvagens do Brasil foram detectados em cachorro-do-mato (Sousa et al., 2017d). Genótipos 16S rRNA de *Anaplasm*a spp. filogeneticamente próximos a *A. bovis* e *A. phagocytophilum* foram detectados em cachorro-do-mato e cães domésticos (*Canis familiaris*) no Pantanal sul-matogrossense (Sousa et al., 2017d). *Anaplasma* spp. (gene 16S rRNA), *Ehrlichia* spp. (gene *dsb*), e *Neorickettsia* spp. (gene 16S rRNA) foram detectados em carrapatos, ácaros e moscas Streblidae de morcegos em Campo Grande, Mato Grosso do Sul (Ikeda et al., 2021). *Ehrlichia* spp., '*Candidatus* Anaplasma brasiliensis' e '*Candidatus* Anaplasma amazonensis' foram detectados em animais da superordem Xenarthra (Ordem Cingulata e

Pilosa – tamanduás, preguiças e tatus) (Calchi et al., 2020). Mais recentemente, um caso incomum de Anaplasmose humana foi relatado na Amazônia, Guiana Francesa, causada por '*Candidatus* Anaplasma sparouinense', filogeneticamente relacionado com genótipos de '*Candidatus* A. amazonensis' previamente detectado em preguiças no Brasil (Duron et al., 2022). Esse relato demonstra a importância da vigilância de animais selvagens quanto à infecção por agentes Anaplasmataceae, principalmente em áreas de contato próximo de seres humanos com vida selvagem.

Estudos prévios mostraram que mamíferos da família Procyonidae pode ser infectados por agentes Anaplasmataceae. Nos Estados Unidos, guaxinins (*Procyon lotor*) são incriminados como importantes reservatórios de *A. phagocytophilum* (Yasbley et al., 2008a; André, 2018). Embora guaxinins sejam encontrados naturalmente infectados por *E. canis* (Dugan et al., 2005), parecem não ser suscetíveis à infecção experimental por *E. canis* e *E. ewingii.* Por outro lado, esses animais mostraram-se suscetíveis à *E. chaffeensis* em infecção experimental (Yabsley et al., 2008a). Em relação à detecção molecular, DNA de *A. bovis* (5,15%) foi detectado em guaxinins no Japão (Sashika et al., 2012), *A. phagocytophilum* em guaxinins nos Estados Unidos (24,6%) (Levin et al., 2002) e Polônia (1,3%) (Hildebrand et al., 2018), e *E. canis* nos Estados Unidos (1,7%) (Dugan et al., 2005) e Espanha (2,6%) (Criado-Fornelio et al., 2018). No Brasil, genótipos 16S rRNA de *A. bovis* (13%), *Anaplasma* sp. filogeneticamente associado a *A. phagocytophilum* (3,2%) e *Ehrlichia* sp. filogeneticamente associada a *E. canis* (3,2%) foram detectados em quatis no Pantanal do estado de Mato Grosso do Sul (Sousa et al., 2017d). *Neorickettsia helminthoeca* foi detectada em amostras de baço e intestino de quatis em Foz do Iguaçu, Paraná, por meio de análises imunohistoquímicas (Headley et al., 2018). Embora DNA do agente tenha sido detectado em fragmentos de tecidos, os autores não conseguiram DNA de boa qualidade para realizar o sequenciamento. Recentemente, DNA de *Ehrlichia/Anaplasma* foi detectado em quatis em Foz do Iguaçu, Paraná, porém os autores não obtiveram sucesso no sequenciamento das amostras (Collere et al., 2021). Devido a informações insuficientes

sobre agentes Anaplasmataceae em Procionídeos no Brasil, estudos objetivando análises moleculares são requeridas.

3.3 Hemoplasmas

Os hemoplasmas (micoplasmas hemotróficos) são bactérias epi-eritrocitárias obrigatórias que infectam grande variedade de hospedeiros mamíferos (Biondo et al., 2009). Anteriormente conhecidos como *Haemobartonella* e *Eperythrozoon* (Messick, 2004), esses agentes foram reclassificados após análises moleculares do gene 16S rRNA de quatro espécies, nomeadamente *Haemobartonella felis*, *Haemobartonella muris*, *Eperythrozoon suis* e *Eperythrozoon wenyonii*, que se mostraram filogeneticamente relacionadas a espécies pertencentes ao gênero *Mycoplasma (*Neimark et al., 2001). As infecções por tais agentes podem se manifestar de duas formas: aguda e crônica. Infecções agudas induzem a anemia e hemólise aguda, e a doença é caracterizada por anorexia, letargia, desidratação, perda de peso e, em alguns casos, morte súbita (Neimark et al., 2001; Willi et al., 2007). A infecção crônica, por outro lado, pode ocorrer em animais aparentemente saudáveis, principalmente aqueles que foram submetidos a procedimento de esplenectomia ou estão imunocomprometidos. A gravidade da doença dependerá da cepa ou espécie de *Mycoplasma* envolvida (Messick, 2004).

As principais vias de transmissão de hemoplasmas parecem ser principalmente por meio de artrópodes hematófagos ou interações agressivas entre animais (Neimark et al., 2001; Macieira et al., 2008; Biondo et al., 2009). Porém, poucos estudos experimentais foram realizados nesse sentido, uma vez que tais agentes ainda não foram cultivados *in vitro* (Biondo et al., 2009). Tem sido sugerida a participação das pulgas *Ctenocephalides felis* na transmissão de *Mycoplasma haemofelis* e '*Candidatus* Mycoplasma haemominutum', bem como o papel dos piolhos *Polyplax serrata* e *Polyplax spinulosa* na transmissão de *Mycoplasma coccoides*, e insetos *Stomoxys calcitrans* e *Aedes aegypti* na transmissão de *Mycoplasma suis* (Biondo et al., 2009). Além disso, infecções naturais em suínos por *M. suis*

têm sido associadas à transmissão mecânica por agulhas e instrumentos cirúrgicos contaminados e por transmissão vertical (Henry, 1979; Dietz et al., 2014; Stadler et al., 2019). A detecção de DNA de *M. suis* no piolho *Haematopinus suis* pode indicar um possível papel desse artrópode como vetor mecânico (Acosta et al., 2019). Apesar de o carrapato *R. sanguineus* ter sido incriminado como vetor e reservatório de *Mycoplasma haemocanis*, essa suposição ainda não foi comprovada experimentalmente (Novacco et al., 2010; Vieira et al., 2015). *Mycoplasma haemofelis* e '*Candidatus* Mycoplasma haemominutum' foram detectados na saliva e glândulas salivares de gatos infectados com hemoplasma, sugerindo que essas bactérias podem ser transmitidas por interações sociais agressivas (Dean et al., 2008).

Hemoplasmas mostram-se como agentes patogênicos importantes para a Saúde Pública, visto que já foram detectados em seres humanos em diversas regiões do mundo, tais como na China (*M. suis*) (Yang et al., 2000; Yuan et al., 2009), Brasil (*M. haemofelis*) (Santos et al., 2008), Inglaterra (*Mycoplasma haemohominis*) (Steer et al., 2011), Estados Unidos (*M. ovis*, '*Candidatus* Mycoplasma haematoparvum') (Sykes et al., 2010; Maggi et al., 2013a), África do Sul ('*Candidatus* Mycoplasma haematoparvum') (Maggi et al., 2013b), Japão ('*Candidatus* Mycoplasma haemohominis') (Hattori et al., 2020) e Nova Caledônia ('*Candidatus* Mycoplasma haemohominis') (Descloux et al., 2020). No Texas, Estados Unidos, Sykes et al. (2010) relataram a ocorrência de co-infecção *por Bartonella henselae* e um hemoplasma intimamente relacionado a *Mycoplasma ovis* em um veterinário com sinais de doença neurodegenerativa progressiva. O paciente relatou histórico de ocorrência de mordidas e arranhões por gatos, cães, pequenos roedores e já havia trabalhado com animais silvestres e zoológicos (Sykes et al., 2010). Infecção por hemoplasma foi relatada em veterinário que apresentou sintomas neurológicos nos Estados Unidos (Maggi et al., 2013a). O paciente referia contato com pulgas, carrapatos, moscas, mosquitos, aranhas, ácaros, além de arranhões ou mordidas de cães, gatos, pássaros, cavalos, répteis, coelhos e roedores. Com base em ensaios de moleculares, foi detectada presença de DNA de *A.*

platys e *Mycoplasma* spp.. As sequências de hemoplasmas obtidas dos genes 16S rRNA e RNaseP apresentaram identidade de 99,8% e 100%, respectivamente, para '*Candidatus* Mycoplasma haematoparvum' (Maggi et al., 2013a). No Brasil, *Mycoplasma haemofelis* foi detectado em um paciente HIV-positivo co-infectado com *B. henselae* (Santos et al., 2008). O paciente apresentava sinais clínicos de sudorese noturna, perda de apetite, tosse produtiva, dores musculares e linfonodomegalia cervical, axilar e inguinal. Neste caso, a participação dos gatos como possível fonte de infecção por esses agentes ao paciente foi sugerida, uma vez que todos os cinco gatos testados, mantidos como animais de estimação pelo paciente, foram positivos para *Bartonella* spp. e dois para *M. haemofelis*. Vieira et al. (2015) encontraram uma amostra positiva para *Mycoplasma* spp. em 100 humanos amostrados (1,0%) no estado do Paraná em ensaio de qPCR para hemoplasmas baseada no gene 16S rRNA. Embora esses achados possam representar casos isolados sem grande importância epidemiológica, são necessários novos estudos para identificar a real importância desses agentes em Saúde Pública.

Em relação a detecção de hemoplasmas em animais selvagens no Brasil, diversos são os relatos de detecção molecular. No Brasil, '*Candidatus* Mycoplasma haematoterrestris' e '*Candidatus* Mycoplasma haematotapirus' foram detectados em antas (*Tapirus terrestris*) (Mongruel et al., 2022). Em animais da Superdem Xenarthra, '*Candidatus* Mycoplasma haematotetradactyla' foi detectado em *Tamandua tetradactyla,* '*Candidatus* Mycoplasma haematomaximus' em *Priodontes maximus*, e genótipo filogeneticamente associado a *Mycoplasma wenyonii* em *Myrmecophaga tridactyla,* (Oliveira et al., 2022). Infecção por espécies de hemoplasmas foram detectadas em carnívoros selvagens da família Felidae de vida livre e cativeiro na América do Sul, Europa e África (Willi et al., 2007). Em São Paulo, 11/110 (10%) animais mostraram-se positivos, sendo *'Candidatus* Mycoplasma haemominutum' detectado em leões, gato-maracajá (*Leopardus wiedii)*, onça parda e gato-do-mato-pequeno (Willi et al., 2007). Em Curitiba e Foz do Iguaçu, um leão e um gato-maracajá, respectivamente (Guimarães, 2008), mostraram-se positivos para *Mycoplasma*

haemofelis. André et al. (2011) encontraram 22 (13%) felídeos brasileiros positivos para '*Candidatus Mycoplasma haemominutum'*, sendo quatro leões, três onças-pardas, dez jaguatiricas, dois gatos-mouriscos e três gatos-do-mato-pequeno. Nesse mesmo estudo, somente um animal da espécie *Leopardus tigrinus* mostrou-se positivo para *Mycoplasma haemofelis* e dois cachorros-vinagre mostraram-se positivos para *Mycoplasma* sp. filogeneticamente relacionado a '*Candidatus* Mycoplasma haematoparvum'.

Em animais da família Procyonidae, em estudo realizado por Volokhov et al. (2017) nos Estados Unidos, 59/95 guaxinins avaliados (62,1%) mostraram-se positivos para micoplasmas hemotrópicos na PCR baseada no gene 16S rRNA e filogeneticamente relacionados a '*Candidatus* Mycoplasma erythrodidelphis', '*Candidatus* Mycoplasma haemozalophi', '*Candidatus* Mycoplasma haemominutum' e '*Candidatus* Mycoplasma haemolamae'. Sousa et al. (2017a) detectaram 24 (77,4%) quatis de vida livre no Pantanal brasileiro positivos para *Mycoplasma* sp. na PCR baseada no gene 16S rRNA e 14 (4,6%) positivos baseado no gene *RnaseP*. As sequências detectadas em quatis mostraram-se filogeneticamente próximas a sequências de *Mycoplasma* sp. detectadas em guaxinins nos Estados Unidos, e *Mycoplasma haemofelis* detectada em gato-do-mato-pequeno (*Leopardus tigrinus*) no Brasil. Ainda, *Mycoplasma* spp. foi detectado em quatis mantidos em cativeiro no sul do Brasil, com base em PCR direcionada ao gene 16S rRNA. As sequências encontradas apresentaram-se filogeneticamente próximas a sequências detectadas em capivaras (*Hydrochaeris hydrochaeris*) amostradas no Brasil (Cubilla et al., 2017). Recentemente, '*Candidatus* Mycoplasma haematonasua', uma espécie de hemoplasma potencialmente nova, foi identificada baseada em análises das sequências dos genes 16S rRNA e 23S rRNA em quatis em Foz do Iguaçu, Paraná (Collere et al., 2021). Devido a importância dos hemoplasmas para a saúde pública e animal, estudos de vigilância de epidemiológica de tais agentes em animais que habitam regiões periurbanas são importantes.

3.4 *Bartonella* sp.

A família Bartonellaceae pertence à Ordem Rhizobiales e compreende alfa-proteobactérias Gram-negativas, intracelulares facultativas, que vêm sendo identificadas em uma ampla variedade de mamíferos e ectoparasitas, incluindo seres humanos (Breitschwerdt et al., 2010; Gonçalves et al., 2016; Fontalvo et al., 2017; Ikeda et al., 2017, 2020; Sousa et al., 2018a; Calchi et al., 2020). A transmissão de patógenos dessa família está associada a vetores artrópodes hematófagos (Breitschwerdt et al., 2010). Com pelo menos 45 espécies já descritas, são consideradas agentes (re)-mergentes e zoonóticos, podendo causar desde manifestações auto-limitantes até enfermidades sistêmicas fatais (Pitassi, 2015; Breitschwerdt et al., 2010; Vieira-Damiani et al., 2016; Okaro et al., 2017). Considerando-se o grande número de animais reservatórios e de artrópodes vetores, a transmissão desses agentes para seres humanos leva a um cenário epidemiológico desafiador (Breitschwerdt, Kordick, 2000; Breitschwerdt et al., 2010).

A transmissão das espécies de *Bartonella* ocorre principalmente por meio de vetores artrópodes, principalmente pulgas e piolhos (Bai et al., 2011; Morse et al., 2012). Porém, pouco se sabe sobre o envolvimento de carrapatos, ácaros e moscas nos ciclos de transmissão do patógeno. A transmissão de *Bartonella bacilliformis* ocorre através de picadas do flebótomo *Lutzomyia verrucarum*, *Bartonella quintana* por fezes do piolho *Pediculus humanus* corporis, e *Bartonella henselae*, *Bartonella clarridgeiae* e *Bartonella koehlerae* por fezes de *Ctenocephalides felis felis* (Chomel et al., 2006; Billeter et al., 2008). Embora espécies de *Bartonella* tenham sido detectadas molecularmente em carrapatos dos gêneros *Ixodes*, *Dermacentor*, *Haemaphysalis* e Argas *(Carios)* (Billeter et al., 2008), a competência vetorial da maioria destas espécies de carrapatos na transmissão de *Bartonella* ainda não foi comprovado experimentalmente (Telford, Wormser, 2010). Alguns trabalhos demonstraram a competência vetorial de carrapatos do gênero *Ixodes* e *Rhipicephalus* na transmissão de *Bartonella* sp.. *Ixodes ricinus* mostrou ser um vetor competente para *B.*

henselae com perpetuação transestadial (Cotté et al., 2008). Os referidos autores observaram a proliferação da bactéria em glândulas salivares de *I. ricinus*, os quais transmitiram com sucesso *B. henselae* ao sangue de sistema de alimentação artificial. Reis et al. (2011) realizaram o primeiro ensaio *in vivo* e demonstraram a competência vetorial de *I. ricinus* na transmissão de *Bartonella birtlesii*. Mais recentemente, Wechtaisong et al. (2020) mostraram que *B. henselae* pode ser transmitida transestadialmente de larvas para ninfas de carrapatos *R. sanguineus* sensu lato. Larvas de carrapatos foram alimentadas com sangue infectado por *B. henselae* através de um sistema de alimentação artificial e testadas em *pools* por PCR após cinco dias. A positividade para *B. henselae* encontrada foi de 57,1% (8/14) em larvas ingurgitadas; 66,7% (4/6) em larvas semi-ingurgitados, e 66,7% (2/3) nos *pools* de fezes de larvas. Após a muda, os *pools* de ninfas também foram testados e 10% (1/10) foram positivos. DNA de *Bartonella* não foi encontrado nas fezes dos carrapatos. Os referidos autores também sugeriram que ninfas podem transmitir a bactéria pela saliva por meio do repasto sanguíneo, uma vez que DNA de *Bartonella*, mas não colônias, foi detectado em amostras de sangue do sistema de realimentação no qual se alimentaram ninfas previamente infectadas no estágio de larva. Recentemente, Wechtaisong et al. (2021) observaram que larvas obtidas de fêmeas ingurgitadas que ingeriram sangue contaminado com *B. henselae* foram capazes de injetar DNA deste agente no sangue não-infectado durante sua alimentação em sistema de alimentação artificial, o que sugere a possibilidade de transmissão transovariana de *B. henselae* em *R. sanguineus* s.l. Além disso, o isolamento de *B. henselae* das fezes de carrapatos adultos enfatiza a possibilidade de que as fezes de carrapatos tenham importância na transmissão de *B. henselae*.

Além da transmissão vetorial, foi descrito que *Bartonella* spp. pode ter outras vias de transmissão. Pitassi et al. (2015) relataram a detecção molecular de *B. henselae* e *B. clarridgeiae* de uma amostra de sangue de doador humano. Embora a transmissão por essa via em humanos ainda não tenha sido confirmada, Silva et al. (2016) demonstraram a transmissão de *Bartonella* por meio de transfusão sanguínea em ratos, sendo que dois em

cada quatro animais transfundidos experimentalmente mostraram-se positivos para *B. henselae* por ensaios moleculares. Acidentes com agulhas contaminadas também podem representar vias adicionais de transmissão (Oliveira et al., 2010; Lin et al., 2011).

Uma das doenças em humanos mais importantes causadas pelo gênero *Bartonella* trata-se da Doença da Arranhadura do Gato (DAC), causada por *B. henselae,* que ocorre mais frequentemente em pacientes imunocomprometidos com histórico de contato com gatos domésticos (Velho et al., 2003). Este agente foi isolado e identificado pela primeira vez em 1992 por Dolan e colaboradores (apud Pitassi, 2013). A infecção por *B. henselae* pode apresentar duas apresentações clínicas principais em humanos: a doença típica, que se caracteriza como uma pápula eritematosa no local do trauma, que se torna vesicular e crostosa, evoluindo para uma mácula com linfadenomegalia; e a doença atípica, caracterizada por febre alta, síndrome oculoglandular de Parinaud, encefalite, anemia hemolítica, hepatoesplenomegalia, glomerulonefrite, pneumonia, bacteremia recidivante e osteomielite (Chomel et al., 2004). Também associada à DAC, *Bartonella clarridgeiae* (Dehio, 2005) pode causar quadros clínicos caracterizados por febre, mal-estar, linfadenopatia e manifestações toracopulmonares em humanos, além de endocardite e hepatopatia em cães, e cegueira e neurite em gatos (Vieira-Damiani et al., 2015). *Bartonella bacilliformis* é o agente causador da doença de Carrión, endêmica no Peru, Equador e Colômbia, com relatos esporádicos na Bolívia, Chile e Guatemala. Duas fases distintas ocorrem no curso da infecção: a primeira, que é conhecida como febre de Oroya, é caracterizada por febre, palidez, desconforto e mialgia; a segunda fase, conhecida como eruptiva ou “verruga peruana”, tem caráter crônico e caracteriza-se clinicamente pela presença de nódulos proeminentes roxo-avermelhados que aparecem geralmente nos membros e na face, sinais neurológicos e cardíacos, com complicações respiratórias em casos graves. *Bartonella quintana* e *B. henselae* são conhecidos como agentes causadores de angiomatose bacilar, que se caracteriza por lesões de proliferação vascular devido à proliferação angiogênica induzida por bartonellae (Velho, 2001). *Bartonella quintana* é

também o agente etiológico da Febre das Trincheiras (Velho, 2001; Pitassi, 2013) e ressurgiu no século XX como uma doença encontrada em grupos sociais em situação de vulnerabilidade, como moradores de rua e dependentes em drogas infestados por piolhos (*Pediculus humanus corporis*) (Dehio, 2004).

Diversos são os relatos de *Bartonella* sp. infectando animais selvagens. Em carnívoros selvagens, DNA de *Bartonella henselae* foi detectada em felídeos das espécies *P. leo, Acynonyx jubatus, P. concolor* e *Lynx rufus* na África (Molia et al., 2004; Pretorius et al., 2004). No Brasil, anticorpos anti-*B. henselae* foram detectados em felídeos mantidos em cativeiro (Filoni, 2006) e espécies de *Bartonella* filogeneticamente relacionadas à *B. koehlerae* e *B. henselae* foram detectadas por PCR em 10/67 (15%) felídeos neotropicais mantidos em cativeiro (Guimarães et al., 2010). Fleischman et al. (2014) detectaram anticorpos anti-*Bartonella* spp. em 11/97 canídeos brasileiros mantidos em zoológicos em São Paulo e no Mato Grosso, sendo anticorpos detectados em 5/39 (12,8%) cachorros-do-mato, 3/27(11,1%) cachorros-vinagre, 2/23 (8,7%) lobos-guarás (*Chrysocyon brachyurus*) e 1/8 (12,5%) raposas-do-campo (*Lycalopex vetulus*). Nos Estados Unidos, DNA de *Bartonella* spp. foi detectado em coiotes (*Canis latrans*) (28%), raposas-vermelhas (*Vulpes vulpes*) (27%) e cangambás (*Mephitis mephitis*) (23%) (Bai et al., 2016). Em animais da família Procyonidae, *B. henselae* e *B. koehlerae* foram detectadas em guaxinins (*Procyon lotor*) nos Estados Unidos (Hwang, Gottdenker 2013; Bai et al., 2016). Até o presente momento, não existem relatos de infecção por *Bartonella* sp. em animais da família Procyonidae no Brasil.

1.5 Piroplasmídeos

Os piroplasmídeos são protozoários intracelulares pertencentes ao Filo Apicomplexa, Ordem Piroplasmida e gêneros *Theileria, Babesia, Rangelia* e *Cytauxzoon* (Yabsley, Shock, 2013; Javlovecka et al., 2018, 2019; Schnittger et al., 2022). Parasitam células sanguíneas de vertebrados, e dependendo do gênero do parasita, podem realizar ciclo em eritrócitos,

linfócitos e monócitos, bem como células endoteliais (Yabsley, Shock 2013; Alvarado-Rybak et al., 2016; Javlovecka et al., 2018). A ordem Piroplasmida é composta por agentes transmitidos por carrapatos que infectam ampla variedade de mamíferos, sendo também relatada em algumas espécies de aves (Alvarado-Rybak et al., 2016). Existem diversas espécies descritas mundialmente, entretanto, esse número tende a aumentar, uma vez que mais espécies animais estão sendo estudadas, indicando que este grupo de protozoários apresenta uma alta diversidade (Criado-Fornelio et al., 2004; Schnittger et al., 2012; Alvarado-Rybak et al., 2016; Solano-Gallego et al., 2016; Javlovecka et al., 2018, 2019; Schnittger et al., 2022). São protozoários de importância econômica por causarem grandes impactos na saúde dos animais domésticos, sendo considerado o segundo grupo de parasitas mais comumente encontrado em mamíferos, logo após *Trypanosoma* (Schnittger et al., 2012). Nos hospedeiros vertebrados, a infecção geralmente é caracterizada por febre, anemia e hemoglobinúria e, em casos severos, pode levar à morte. *Babesia divergens* e *Babesia microti* já foram detectadas em casos fatais de infecções em humanos (Gorenflot et al., 1998, Homer et al., 2000, Herwaldt et al., 2003), sendo o primeiro caso de óbito decorrente de *Babesia* descrito em 1956 (Skrabalo, Deanovic, 1957). No Brasil, casos de babesiose humana já foram relatados nos estados de Pernambuco (descrição de formas evolutivas de *Babesia* spp. em esfregaços sanguíneos) e São Paulo (anticorpos contra *Babesia bovis* em dois pacientes) (Alecrim et al., 1983; Yoshinari et al., 2003).

Nos últimos anos, com o advento da biologia molecular, houve um aumento expressivo de estudos reportando infecções por piroplasmídeos em animais selvagens (Alvarado-Rybak et al., 2016). As infecções por piroplasmídeos são prevalentes em carnívoros selvagens no mundo todo, embora existam poucas informações sobre a importância epidemiológica e clínica da babesiose neste grupo de mamíferos. De fato, *Babesia* spp. foram detectadas em várias espécies de carnívoros das famílias Canidae, Felidae, Herpestidae, Hyaenidae, Mustelidae, Viverridae, Ursidae e Procyonidae (Penzhorn 2006, Alvarado-Rybak et al., 2016). No Brasil, existem poucos relatos da detecção sorológica e molecular de *Babesia*

spp. em carnívoros selvagens. André et al. (2011b) encontraram soroprevalência para *B. vogeli* de 31,7% e 10,3% em felídeos e canídeos selvagens mantidos em cativeiro, respectivamente. Nesse mesmo estudo, um genótipo filogeneticamente próximo a *B. leo* foi detectado em um gato-palheiro (*Oncifelis colocolo*) e em uma geneta (*Genetta tigrina*), também mantidos em cativeiro em zoológicos do estado de São Paulo. Sousa et al. (2017b) detectaram DNA de *Babesia* filogeneticamente associado a *Babesia caballi* em 1/78 (1,3%) cachorros-do-mato de vida livre amostrados no Pantanal. Em animais da família Procyonidae, espécies de *Babesia* têm sido descritas em *P. lotor* no Japão e Estados Unidos, *N. narica* na Costa Rica e em *P. cancrivorus* no Uruguai (Kawabuchi et al., 2005; Birkenheuer et al., 2006; Birkenheuer et al., 2008; Jinnai et al 2009; Clark et al., 2012; Mehrkens et al., 2013; Thompson et al., 2018). Interessantemente, o agente detectado em *P. lotor* nos Estados Unidos e Japão é filogeneticamente distinto daquele detectado em *P. cancrivorous* no Uruguai, sugerindo uma diversidade de espécies de piroplasmídeos encontradas em procionídeos. *Procyon lotor* nos Estados Unidos e Japão parecem atuar como reservatórios de *Babesia* sp. e podem participar da dinâmica das infecções por *B. microti* em humanos dispersando carrapatos *Ixodes* sp. infectados (Jinnai et al 2009; Clark et al., 2012). No Brasil, não existem até o momento relatos sobre infecções por *Babesia* spp. em *N. nasua*.

Os protozoários do gênero *Theileria* infectam uma ampla variedade de hospedeiros, tanto domésticos quanto selvagens, e são transmitidas por carrapatos ixodídeos dos gêneros *Amblyomma, Haemaphysalis, Hyalomma* e *Rhipicephalus* (Bishop et al., 2004). Poucos são os relatos da detecção de *Theileria* em carnívoros selvagens no mundo. Duas espécies de *Theileria* (*T. sinensis* e *T. parva*) foram molecularmente detectadas em amostras de sangue de leões mantidos em cativeiro na África (Kelly et al., 2014). Githaka et al. (2012) detectaram piroplasmídeos filogeneticamente associados a *Theileria* spp. previamente descritas em ovelhas e girafas em guepardos (*Acinonyx jubatus*) amostrados no Quênia. Na Alemanha, 44/261 (16,85%) das raposas (*V. vulpes*) analisadas

mostraram-se positivas para *Theileria annae* (atualmente nomeada como *Babesia vulpes*) (Najm et al., 2014; Baneth et al., 2015). No Brasil, DNA de *Theileria* sp. foi detectado em 3/31 (9,67%) quatis amostrados no Pantanal, e as sequências mostraram 100% de identidade com uma amostra de *Theileria* sp. detectada previamente em um felino doméstico no Brasil, filogeneticamente relacionado ao clado de *Theileria equi* (Sousa et al., 2017b). Devido ao pequeno número de relatos sobre piroplasmídeos em carnívoros selvagens no Brasil e a falta de informações sobre sua importância em Saúde Pública, epidemiologia e formas de transmissão, estudos devem ser conduzidos buscando a detecção e caracterização molecular desses agentes.

1.6 *Rickettsia* spp. em carrapatos associados a procionídeos

Bactérias do gênero *Rickettsia* (Rickettsiales: Rickettsiaceae) pertencem à classe das alfa-proteobactérias e são agentes Gram-negativos intracelulares obrigatórios, tendo como principal alvo as células endoteliais, sendo transmitidas por uma variedade de artrópodes, principalmente pulgas e carrapatos (Parola et al., 2005; Schroeder et al., 2015). O gênero *Rickettsia* inclui diversas espécies validadas, e um número cada vez maior de genótipos novos (Parola et al., 2013). Alguns dos agentes recém-identificados provaram causar doenças humanas emergentes, tais como *Rickettsia aeschlimannii* descrita em um viajante que retornou do Marrocos (Raoult, Fournier 2002), *Rickettsia parkeri* na Virginia, EUA (Paddock et al., 2004) e *Rickettsia amblyommatis*, suspeita de causar doença na Carolina do Norte, EUA (Apperson et al., 2008). A percepção da importância das doenças causadas por espécies de *Rickettsia* em saúde pública vem crescendo, pois estão associadas a vetores artrópodes (carrapatos, pulgas, piolhos e ácaros) com ampla distribuição mundial, mantidos em ambientes florestais por hospedeiros que atuam como amplificadores da bactéria.

O gênero *Rickettsia* é dividido em quatro grupos de acordo com padrões antigênicos, morfológicos, moleculares e ecológicos: 1) o grupo Tifo, que compreende as espécies *Rickettsia prowazekii* e *Rickettsia typhi*, associadas a piolhos e pulgas, respectivamente; 2) o grupo da febre maculosa (Spotted Fever Group - SFG), composto por espécies como *Rickettsia rickettsii, Rickettsia parkeri* e *Rickettsia africae*, a grande maioria associada a carrapatos; 3) o grupo *Rickettsia canadensis*, com *R. canadensis* transmitida por carrapatos; e 4) o grupo *Rickettsia bellii*, composto por *R. bellii* e outras espécies simbióticas encontradas em carrapatos (Labruna 2009; Merhej, Raoult, 2011; Bhengsri et al., 2016). No Brasil, bactérias do gênero *Rickettsia* causam pelo menos duas doenças em seres humanos. O Tifo murino do grupo do tifo exantemático, causado por *R. typhi*, já foi descrito nos estados de Minas Gerais (Silveira, Maestrini 1985), São Paulo (Anadão 1954) e Rio de Janeiro (Pereira et al., 1949). A febre maculosa brasileira (FMB) é causada por *R. rickettsii* e transmitida por *A. aureolatum* e *A. sculptum* (complexo *Amblyomma cajennense*) (Szabó et al., 2013a). Há uma década, uma febre maculosa mais branda foi descrita por Spolidorio et al. (2010), causada por *Rickettsia parkeri* cepa Mata Atlântica e transmitida por *A. ovale* e *A. aureolatum* (Szabó et al., 2013b). Outra espécie de *Rickettsia* já detectada no Brasil é *Rickettsia belli*, que até o presente momento tem patogenicidade desconhecida, pois não foi detectada em humanos ou outros animais. É considerada a espécie mais primitiva do gênero, além de ter alta prevalência em populações de *Amblyomma* spp. Acredita-se ser uma espécie simbiótica, com possíveis processos de coevolução com os carrapatos hospedeiros (Labruna et al., 2004a; McIntosh et al., 2015). Recentemente, anticorpos contra *R. typhi* (titulação 2.048 para Wilmington *strain*) foram dectados em um paciente com febre, dor de cabeça e tosse produtiva, no estado do Pará, sendo o primeiro relato da doença fora dos estados de São Paulo, Rio de Janeiro e Minas Gerais (Minervino et al., 2020).

Diversos estudos vêm sendo realizados visando a detecção molecular de bactérias do gênero *Rickettsia* em carrapatos no Brasil. Um total de 3.545 carrapatos *A. sculptum* e 2.666 *A. dubitatum* provenientes de 16 localidades do Estado de São Paulo foram coletados

em vida livre e, também através da retirada manual sobre as capivaras. Os carrapatos foram submetidos à PCR para *Rickettsia* spp. baseada no gene *gltA.* Todos os espécimes de *A. sculptum* foram negativos, e 634 (23,8%) *A. dubitatum* mostraram-se infectados com *Rickettsia bellii* (Pacheco et al., 2009). Soares e colaboradores (2015) estudando carrapatos amostrados de animais selvagens caçados nos estados do Mato Grosso e Pará, detectaram *'Candidatus* Rickettsia amblyommatis' em quatro espécies de carrapatos *A. cajennense*, *Amblyomma auricularium, Amblyomma longirostre* e *Amblyomma humerale; R. bellii* foi detectada em *A. humerale* e *Amblyomma naponense; Rickettsia rhipicephali* foi detectada em *H. juxtakochi* e *Rickettsia felis* em *A. humerale*. Duas possíveis novas espécies de *Rickettsia* foram detectadas em carrapatos *A. naponense*, sendo um novo genótipo do grupo da febre maculosa filogeneticamente relacionado com *R. africae* no Pará, e um novo genótipo do grupo *Canadensis* no Mato Grosso. Sousa e colaboradores (2018b) analisando 523 amostras de carrapatos coletados de mamíferos do Pantanal, obteve 116 (23%) amostras positivas para *Rickettsia* sp. em uma qPCR baseada no gene *ompA* (93 *A. parvum*, 14 *A. sculptum*, 3 *Amblyomma auricularium* e 6 "pools" de larvas de *Amblyomma* spp. Trinta amostras mostraram-se positivas na PCR convencional e foram sequenciadas, apresentando 100% de identidade com '*Candidatus* Rickettsia andeanae' (Sousa et al., 2018b). Magalhães-Matos e colaboradores (2022) testaram 566 carrapatos coletados de quatis amostrados no Parque Nacional do Iguaçu (larvas de *Amblyomma* spp., *H. juxtakochi, A. brasiliense, A. coelebs* e *A. ovale)*. Espécimes de *A. coelebs* (1,90%, 8/420) foram positivas para *Rickettsia amblyommatis,* enquanto espécimes de *A. ovale* (13%, 6/45) foram positivas para *R. bellii*. A despeito da grande diversidade de carrapatos que já foi descrita parasitando quatis, até o presente momento apenas um único trabalho relatou a presença de *Rickettsia* spp. em ectoparasitas coletados desse grupo de animais (Magalhães-Matos et al., 2022). Desta forma, são necessários mais estudos objetivando investigar a presença de rickéttsias em carrapatos de quatis no Brasil, principalmente aqueles que habitam áreas peri-urbanas.

Filarídeos

Entre as parasitoses transmitidas por vetores estão as filarioses, que apresentam distribuição mundial e são originadas pela infecção por helmintos da família Onchocercidae. Possuem um ciclo de vida indireto e os vetores conhecidos, mosquitos e carrapatos, estão amplamente distribuídos em todo o mundo (Cancrini et al., 2003; Laaksonen et al., 2009; Otranto et al., 2012; Brilhante et al., 2020; Netherlands et al., 2020). Os helmintos adultos podem ser encontrados em diferentes órgãos, desde olhos, tecido subcutâneo, cavidades corporais e órgãos importantes como coração e pulmões (Orihel, Eberhard, 1998; Pampiglione et al., 2001; Morchón et al., 2012; Magnis et al., 2013).

Entre os gêneros da família Onchocercidae, o mais conhecido mundialmente é *Dirofilaria*, devido a distribuição mundial da espécie *Dirofilaria immitis* e distribuição na Europa, Ásia e África de *Dirofilaria repens*, agentes etiológicos da dirofilariose canina e dirofilariose subcutânea, respectivamente (Genchi, Kramer 2017; Otranto, Deplazes, 2019).

O primeiro registro de *D. immitis* na América do Sul foi realizado em 1878, no Estado da Bahia, Brasil (Silva et al., 1978). Os helmintos adultos parasitam o ventrículo direito e frequentemente o tronco principal das artérias pulmonares do hospedeiro, determinando endarterite, alterações no fluxo sanguíneo e na viscosidade do sangue. É encontrada principalmente no cão doméstico (Anvari et al., 2020), porém já foram descritas infecção em gatos domésticos (Travassos 1921; Meireles et al., 2001) e em carnívoros selvagens (Penezic et al., 2014; Panayotova-Pencheva et al., 2016). Existem atualmente mais de 72 espécies de culicídeos descritas como vetores de *D. immitis* no mundo todo, distribuídas entre os gêneros *Culex, Aedes, Ochlerotatus, Anopheles, Mansonia, Psorophora e Coquillettidia* (Lozovei, 2001; Vezzani et al., 2006; Morchón et al., 2012).

Dirofilaria repens causa geralmente nódulos subcutâneos não-patogênicos em canídeos domésticos e selvagens, e é o principal agente da dirofilariose humana no Velho

Mundo (McCall et al., 2008; Otranto et al., 2015; Capelli et al., 2018). Em seres humanos, os estágios de desenvolvimento de *D. repens* migram por via subcutânea por semanas até vários meses em várias partes do corpo, geralmente com sintomas leves e muitas vezes despercebidos e apenas algumas vezes causando sintomas semelhantes à *larva migrans*, ou seja, irritação e prurido (Pampiglioni, Rivasi 2000; Pampiglioni et al., 2001; Vries et al., 2003; Pampiglioni et al., 2008). Durante a migração das larvas, *D. repens* pode atingir os olhos, tornando-se visível através da subconjuntiva, causando dor e inchaço na região ocular (Pampiglioni et al., 2001; Otranto, Eberhard 2011; Frenzen et al., 2020).

Outras espécies do gênero já foram relatadas em infecções humanas, tais como *Dirofilaria ursi*, que tem como hospedeiros o urso-preto-americano (*Ursus americanus*) e o urso preto-japonês (*Ursos thiabetanus japonicus*) (Uni et al., 1980; Yamada et al., 2017), *Dirofilaria subdermata*, que infecta porcos-espinhos (*Erethizon dorsatum*) da região noroeste dos Estados Unidos e Canadá (Gutierrez, 1983), além de *Dirofilaria tenuis*, parasita de guaxinins (*Procyon lotor*) nos Estados Unidos (Isaza, Courtney 1988). Na medicina humana, as filarioses mais estudadas são a Filariose brancroftiana, causada por *Wuchereria bancrofti*, as filarioses linfáticas africanas e asiáticas, causadas por *Brugia timori, Brugia pahangi, Brugia malayi, Loa loa, Onchocerca volvulus* (WHO, 2010; Ramaiah, Ottesen, 2014), *Mansonella perstans* e *Mansonella streptocerca*, além da *Mansonella ozzardi,* a qual ocorre apenas na América Latina, sendo a região amazônica endêmica para esta filariose no Brasil (Lima et al., 2016; Silva, 2016; Medeiros et al., 2009; Medeiros et al., 2017; Tang et al., 2018).

No Brasil, foram descritos 26 gêneros com 91 espécies de filarídeos parasitando desde anfíbios até humanos (Vicente et al., 1997; Vieira et al., 2008). Em carnívoros selvagens brasileiros, foram descritas *Dirofilaria incrassata* parasitando tecido subcutâneo de *N. nasua* e *P. cancrivorus* (Vicente et al., 1997); *D. repens* em tecido subcutâneo de *C. thous* e *N. nasua* (Noronha et al., 2002); *Dirofilaria spectans* em pulmões e grandes vasos de *Eira barbara*, *Lontra longicaudis* (Noronha et al., 2002), *Pteronura braziliensis* (Vicente et

al., 1997), além de *Dirofilaria striata* parasitando *L. wiedii* e *P. concolor* (Lent, Freitas, 1937; Vicente et al., 1997). Adicionalmente, foram descritos filarídeos dos gêneros *Brugia* sp*., Dirofilaria* sp*., Acanthocheilonema* sp. e *Mansonella* sp. em *N. nasua* no Parque Nacional do Iguaçu (Moraes et al., 2017; 2022). Devido à diversidade de insetos vetores, diversidade de gêneros que podem infectar animais selvagens e o ser humano, e escassez de estudos sobre Onchocerca em animais da família Procyonidae, são importantes estudos objetivando a detecção e caracterização dos referidos agentes.

4. Objetivos

4.1 Objetivos gerais

O presente estudo teve como objetivo investigar a diversidade genética de agentes transmitidos por artrópodes (Anaplasmataceae, Rickettsiaceae, Mycoplasmataceae, Bartonellaceae, Babesiidae/Theileriidae, Hepatozoidae e Onchocercidae) em quatis e/ou seus ectoparasitas amostrados em região periurbana de Campo Grande (MS, Brasil), por meio de um estudo longitudinal.

4.2 Objetivos específicos

- Identificar ectoparasitas em quatis amostrados em região peri-urbana da cidade de Campo Grande;
- Investigar, por meio de métodos moleculares e morfológicos, a ocorrência de agentes das famílias Anaplasmataceae, Mycoplasmataceae, Bartonellaceae, Rickettsiaceae, Theileridae/Babesiidae, Hepatozoidae, e Onchocercidae em amostras de sangue de quatis e/ou seus respectivos ectoparasitas amostrados em região peri-urbana de Campo Grande;

- Isolar *Bartonella* spp. a partir de amostras de sangue de quatis amostrados em região peri-urbana da cidade de Campo Grande, utilizando meios de cultivo líquido e sólido;
- Traçar inferências filogenéticas com genótipos dos agentes sob estudo circulantes em quatis e ectoparasitas amostrados;
- Investigar possíveis alterações clínicas e hematológicas nos quatis amostrados em Campo Grande positivos nas técnicas moleculares para hemoparasitas.

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Capítulo 2* - Diversity and Seasonal Dynamics of Ticks on Ring-Tailed Coatis *Nasua nasua* (Carnivora: Procyonidae) in Two Urban Areas from Midwestern Brazil*.

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Simple Summary: The knowledge of the dynamics of ticks in wild animals is essential for surveil- lance of tick-borne diseases. Coatis (*Nasua nasua*) are mammals that easily adapt to anthropized areas, favoring close contact with domestic animals and humans, favoring the exchange of ticks and tick-borne agents. The present study aimed to investigate the tick diversity on coatis from forest urban areas of midwestern Brazil, as well as the dynamics of ticks during the seasons of the year and the correlation between tick species and gender and age of the sampled coatis. Three tick species were identified parasitizing coatis from forested urban fragments, namely *A. dubitatum* nymphs, *A. sculptum* adults and nymphs, and *A. ovale* adults. After analyzing the obtained results, it is likely that coatis from anthropized areas present tick species diversity lower than those from natural landscapes. The mean intensity and prevalence of *Amblyomma* larvae and nymphs is similar among males and females as well as in immature and mature animals, which might reflect the gregarious behavior of coatis, since adult males live together with females and offspring outside and inside the mating season, forming large groups of individuals.

Abstract: Understanding the diversity and ecology of ectoparasites in wild animals is essential for surveillance of vector-borne diseases. Coatis (*Nasua nasua*) easily adapt to anthropized areas, favoring close contact with domestic animals and humans, with the possibility of exchange of ectoparasites and pathogens. The present study aimed to identify the diversity of ticks parasitizing coatis from forest urban areas of midwestern Brazil, to

evaluate the seasonal dynamics of ticks during the seasons of the year, and to assess the correlation between tick species and gender and age of the sampled coatis. For this purpose, 103 coatis were captured in two Conservation areas, both located in Campo Grande city, Mato Grosso do Sul state, Midwestern Brazil. The animals' entire body was inspected for the presence of ectoparasites, and ticks were removed for taxonomic identification. In total, 168 captures were performed in both areas during the observational study considering the first capture and recaptures. In total, 2242 ticks were collected: 838 *Amblyomma* larvae, 1241 *A. sculptum* nymphs, and 150 *A. dubitatum* nymphs. Thirteen adult ticks were identified as three males and five females of *A. sculptum* and two males and three females of *A. ovale.* While a quantity of *Amblyomma* larvae was observed in the first months of the year (January, April and May), *Amblyomma* nymphs showed a higher quantity during the months of July, August, October and November. No statistical difference was observed when comparing mean intensity and prevalence of *Amblyomma* larvae, nymphs of *A. sculptum* and *A. dubitatum* between the two sampled areas, males vs. females and immature vs. mature animals. In conclusion, three tick species were identified parasitizing coatis from forested urban fragments in midwestern Brazil, namely *A. dubitatum* nymphs, *A. sculptum* adults and nymphs, and *A. ovale* adults. Coatis from anthropized areas seem to present tick species diversity lower than those from natural areas. The lack of statistical difference regarding mean intensity and prevalence of *Amblyomma* larvae and nymphs between males vs. females and immature vs. mature animals might have reflected the gregarious behavior of coatis, since adult males live together with females and offspring outside and inside the mating season, forming large groups of individuals.

Key-words: ectoparasites; *Amblyomma sculptum*; *Amblyomma dubitatum*; *Amblyomma ovale*

1. Introduction

Ring-tailed coatis (*Nasua nasua*) are medium-sized animals belonging to the order Carnivora and the family Procyonidae [1]. Such mammals have a wide geographic distribution in South America, and easily adapt to different environments, especially urbanized areas [2]. This plasticity favors close contact with domestic animals and humans, with the possibility of exchange of ectoparasites and associated pathogens [3]. Carnivores are considered the first source of human infections with zoonotic agents, which include tick-borne pathogens [4–6]. Tick-borne pathogens have already been detected in coatis and associated ectoparasites from Brazil [7–10]. *Theileria* sp., *Anaplasma bovis*, *Anaplasma* sp. closely related to *A. phagocytophilum Ehrlichia* sp., and hemoplasmas were detected in coatis from Pantanal wetland, Mato Grosso do sul state, central-western Brazil [7–9]. Recently, *Rickettsia bellii* and *Rickettsia amblyommatis* were detected in *Amblyomma ovale*

and *Amblyomma coelebs*, respectively, from coatis sampled in Iguazu National Park, Iguazu Falls, Paraná state, southern Brazil [10]. Therefore, the constant surveillance of species, diversity and ecology of ectoparasites in wild animals is essential for monitoring vector-borne diseases.

The Brazilian tick fauna is currently comprised of 75 species, including 24 Argasidae species and 51 Ixodidae species [11–14]. Within the Ixodidae family, the genus *Amblyomma* is the most representative, with 33 species described [11,12,15]. The *Amblyomma* genus is generally composed of three-host tick species, except for *Amblyomma rotundatum* that may present a two-host behavior in snakes and chelonians [16,17] and three-host behavior when fed on frogs [18]. Many tick species present a life cycle of one generation per year with most adults during warmer months, such as *Amblyomma* sp. [19–23]. Depending on tick species, they may present questing or nidicolous behavior. *Amblyomma* spp. are questing ticks, which climb on to vegetation to wait for the passing host (ambush ticks) [24,25]. *Amblyomma sculptum* also exhibits a hunter behavior, where some specimens can emerge from their refuges and run across the ground to find the host. This tick species has a highly aggressive behavior, and it is considered a major human biting tick species in Brazil [23]. Even though *Amblyomma ovale* is also considered a questing tick, a specific population of this tick species exhibited a nidicolous behavior, with all three stages feeding on dogs in an anthropized area in Peruibe in southeastern Brazil [26].

Previous studies assessing the diversity of ectoparasites on coatis from Brazil have showed infestation by *Amblyomma brasiliense* and *Amblyomma ovale* in Paraná state [27]; *A. ovale*, *Amblyomma parkeri*, *A. brasiliense, Amblyomma* spp., *Rhipicephalus (Boophilus) mi- croplus, Rhipicephalus sanguineus* sensu lato (both *Rhipicephalus* from domestic animals) and *Amblyomma sculptum* in Mato Grosso [3]; São Paulo [28–30], Paraíba [31] and Minas Gerais states [28,32] and *A. rotundatum* in Pará state. In a study carried out in the Brazil- ian Pantanal, Mato Grosso do Sul state, coatis were found to be infested by *A. sculptum*, *Amblyomma parvum*, *A. ovale*, *R. (B.) microplus* and *Amblyomma* spp. [7–9]. In the state of São Paulo, in the cities of Botucatu and Palmital, infestations by *Amblyomma dubitatum*, *A. ovale*, *A. sculptum* and *R. sanguineus* s. l. were reported [33]. Gaining knowledge on the diversity and seasonal dynamics of tick species that parasitize free-ranging animals living in urban forest fragments is important to the surveillance of tick-borne diseases in order to establish control measures and also to support the conservation of species of wild animals. Herein, we hypothesized that the diversity of tick species found in anthropized areas is lower than in natural landscapes. The aims of the present study were: (1) to identify the ticks found parasitizing coatis from

urban areas of midwestern Brazil; (2) to evaluate the seasonal dynamics of ticks found parasitizing coatis; and (3) to assess the association between tick species and gender and age of the sampled coatis.

2. Material and Methods

2.1 Sampling Area

Ring-tailed coatis (*N. nasua*) were captured in two Conservation areas, both located in Campo Grande city, Mato Grosso do Sul state, in midwestern Brazil. The phytophys- iognomy of Campo Grande is Cerrado, characterized by a tropical savanna composed by areas of grassland to a nearly closed canopy of medium height trees overlying grass. Campo Grande has a tropical savanna climate, with semi-humid, hot summers, and notably seasonal, with marked dry weather from April to October and rainy weather from November to March [34]. The two samplings' spots in Campo Grande were "Parque Estad- ual do Prosa" (PEP) (-20.44987, -54.56529) and "Vila da Base Aérea" (VBA) (-20.47163, -54.65405) (**Figure 1**).

PEP is a state National Conservation Park 134 ha in size, representing one of the last remnants of the Cerrado biome within the urban perimeter. It preserves regional species of fauna and flora threatened by extinction. The area has become a tourist spot for visitors from cities in the state of Mato Grosso do Sul as well as from different regions of Brazil, so there is a high daily circulation of people (approximately 2000 visitors attend the PEP during the week, and on weekends, it can exceed 6000 users) [34]. It is an important refuge for wildlife, where many species of mammals can be found, such as ring-tailed coatis (*N. nasua*) [34,35], anteaters (*Myrmecophaga tridactyla* and *Tamandua tetradactyla*) [34], capybaras (*Hydrochoerus hydrochaeris*) [34] opossums (*Didelphis albiventris*) [36–38], bats (e.g., *Artibeus* sp. and *Myotis nigricans* [39], birds (e.g., Cuculidae and Psittacidae) and reptiles (*Tupinambis* spp.) [34]. Also, this park has a wild animal rehabilita- tion center, which usually receives free-living animals belonging to several species (available at: https://www.imasul.ms.gov.br/gestao-de-unidades-de-conservacao/unidades- de-conservacao-estaduais/parque-estadual-do-prosa-pep/ Accessed on 19 November 2021). In addition to wildlife, domestic cats and dogs have been seen on site. Two density studies were performed at PEP with coatis, where 33.7 individuals/km^2 were estimated in 2009, equivalent to 120 coatis in the study area [13] and 11.2 individuals/Km^2 in 2021 (30 coatis in the study area) [14].

VBA is a Brazilian Air Force base with an area of approximately 484 ha that presents fragments of Cerrado forest, but no visitors are allowed. It's a residential area, surrounded by three forest fragments and one area used for military training. At least 730 people and their domestic animals inhabited the residential complex. The houses are not fenced, and they all had a trash can at the front. Coatis have access to the outside of the houses, and they were observed during the captures walking around the houses and also searching for food in the dumpsters. Only one density study was performed at VBA, where 19.4 individuals/km^2 were estimated, equivalent to 41 coatis in the study area [14]. Differences between both sampling areas are shown in the text and **Table 1**.

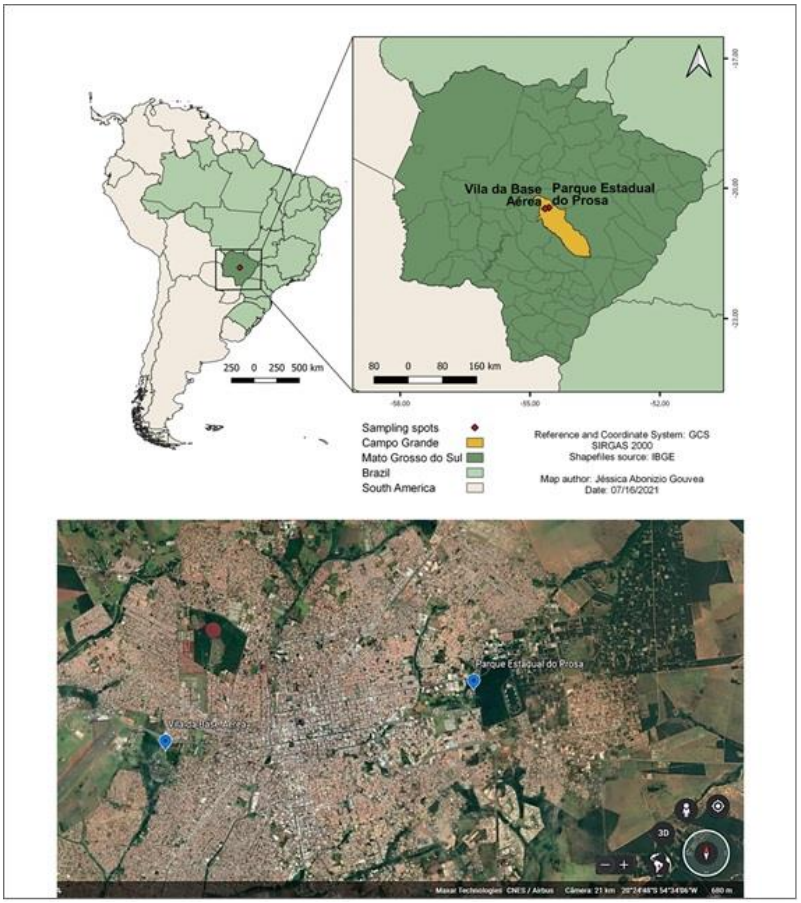

Figure 1. Map showing the state of Mato Grosso do Sul (dark green), with Campo Grande city highlighted in yellow. Source: QGIS Development Team, 2020. QGIS Geographic Information System. Open-Source Geospatial Foundation Project. http://qgis.osgeo.org Accessed on 10 November 2021.

2.2 Capture of the animals

Ring-tailed coatis were captured every three weeks for 10 consecutive days (with intervals of two days—Saturday and Sunday) between March of 2018 and April of 2019. All captures and recaptures were performed by convenience, and dates from recaptures were

by chance. Animals were captured using metal traps (1m x 0.40m x 0.50m) placed arbitrarily according to the possibility of human access and availability of shadow, covering most of the PEP and VBA areas. Captured animals were anesthetized with an association of Tiletamine hydrochloride and Zolazepam hydrochloride (Telazol, Zoetis® (Parsippany-Troy Hills, NJ, USA) (6 mg/kg, intramuscularly) [34]. After chemical restraint, animals were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue between the shoulder blades in order to identify them for future recaptures. Animals were measured and the age was estimated according to Olifiers et al. [40]. The animals' entire body was inspected for the presence of ectoparasites for three minutes. Ticks were removed with the aid of a forceps and stored in 100% alcohol (Merck Ensure®, Darmstadt, Germany)- containing RNAse/DNAse free microtubes. Taxonomic identification of ectoparasites was performed with a stereoscopic microscope (Olympus SZX7, Tokyo, Japan) following taxonomic literature [11,41]. While larvae were identified to genus level, nymphs and adults were identified to species level. All the specimens examined were deposited in the Acari Collection of the Instituto Butantan, São Paulo, Brazil (IBSP) (**Figures S1–S7**).

Table 1. Differences between the two urban fragments from Campo Grande city, Mato Grosso do Sul state, central-western Brazil, where coatis (*Nasua nasua*) where sampled between March of 2018 and April of 2019.

Characteristics	Parque Estadual do Prosa (PEP)	Vila da Base Aérea (VBA)
Geographical coordinates		
Type of sampling area	National Conservation Park	Brazilian Air Force base
Sampling area size	135 ha	484 ha
Estimated coatis' density	33.7 individuals/km^2 in 2009 (120 animals) [13] and 11.2 individuals/km^2 in 2021 (30 animals) [14]	19.4 individuals/km^2 (41 animals) in 2021 [14]
Visitors allowed	Yes	No
Residential área	No	Yes
Presence of domestic cats/dogs	Yes	Yes
Wild animal rehabilitation center	Yes	No

2.3 Statistical analyses

The prevalence was obtained by calculating number of infested coatis divided by the total of sampled coatis. An analysis of the association between the dichotomous dependent variables was performed (presence/absence of ectoparasites (total ticks—all stages; larvae and nymphs) and categorical independent variables (sampling spot (PEP vs. VBA), gender (female vs. male) and age group (immature vs. mature) to detect variables with significant association ($p < 0.05$) in the X^2 test using the Epi Info software [42]. Values of odds ratio and superior and inferior confidence interval were obtained. The intensity (range of infestation: minimum and maximum) and mean intensity (MI) (total number of ticks (larvae, nymphs and adults ÷ number of infested coatis] were assessed. All observations (terms) were defined according to Margolis et al. [43]. For seasonal analyses (months of the year and dry vs. weather season), all data collected was analyzed independently of gender, age or sampling spot. Adult ticks were not included in the statistical analyses due to the small number of specimens sampled. General linear mixed effects models (GLMM) were performed to test if there was an effect of the fixed factors (locality, gender, and age, separately and interactions) in the quantity of ticks to detect factors with significant association ($p < 0.05$) using Glimmix at Statistical Analysis System (SAS). In order to identify seasonal patterns, the number of tick specimens collected in each month were analyzed separately from 2018 and 2019. For statistical analyses regarding sampling spots, gender and age, only the first capture was assessed, since recaptures were performed in different periods of the year (by convenience). For seasonal analyses, all data collected was analyzed independently of gender, age or sampling spot, but data collected from 2018 were analyzed separately from those obtained during 2019. Mean quantity of larvae and nymphs ticks (Total number of collected ticks number of total sampled coatis) were used to evaluate distribution of sampled ticks during months of the year. Information regarding temperature and precipitation in Campo Grande city during 2018 and 2019 were collected from the governmental website of “Centro de Monitoramento do Tempo e Clima de Mato Grosso do Sul” (https://www.cemtec.ms.gov.br/boletins-meteorologicos/ Accessed on 14 January 2022).

3. Results

3.1 Sampling Ring-Tailed Coatis

In total, 168 captures were performed in both areas during the observational study, including the first capture and one to three recaptures (69 in PEP and 99 in

VBA). In PEP, samples were obtained from 48 different coatis (30 females and 18 males; eight cubs, one subadult, and 39 adults). One capture (without recapture) was performed in 32/48 individuals, two captures in 12/48 coatis (first sampling and one recapture), 3/48 animals were recaptured twice (first sampling and two recaptures), and only 1/48 animals was recaptured three times (first sampling and three recaptures), totaling 69 captures in PEP. In VBA, samples were obtained from 55 different individuals (33 females and 22 males; 10 cubs, seven subadults and 38 adults). One capture was performed in 33/55 individuals (one sampling), two captures were performed in 11/55 coatis (first sampling and one recapture), 4/55 were recaptured twice (first sampling and two recaptures), 5/55 were recaptured three times (first sampling and three recaptures) and 2/55 were recaptured five times (first sampling and five recaptures), totaling 99 captures from VBA.

3.2 Ticks Specimens Collected from Ring-Tailed Sampled Coatis

In total, 2242 ticks were collected from coatis in the present study in both areas, includ- ing animals at the first capture and in the recaptures (considering 168 captures). Regarding tick species, 838 larvae were identified as *Amblyomma* spp. Among 1391 nymphs, 1241 were identified as *A. sculptum* and 150 to *A. dubitatum*. Co-infestation by both *A. sculptum* and *A. dubitatum* nymphs were observed in 36 animals. Thirteen adults were identified as three males and five females of *A. sculptum* and two males and three females of *A. ovale*. Regarding adult ticks, only one coati (VBA 27) was co-infested, with two adults belonging to different tick species, represented by one male *A. sculptum* and one male *A. ovale*. Eleven coatis were infested by only one adult tick (four coatis with one *A. ovale* each and seven coatis with one *A. sculptum* each). Ticks were collected from all regions of the body of the animals (**Figure 2A**), either engorged or unengorged, especially in the paws (between fingers), around the eyes and lips, ear tip and genital areas (around foreskin and vulva). Interestingly, one nymph of *A. sculptum* was removed from inside the mouth during physical examination, and the specimen was feeding on the palate (**Figure 2B**).

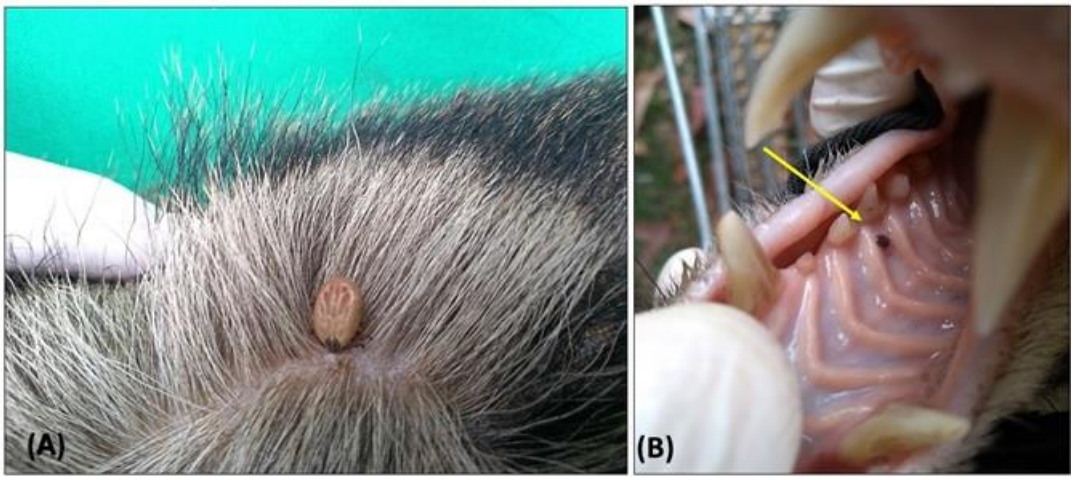

Figure 2. Ticks collected from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil. (**A**) Partially engorged female of *Amblyomma ovale* feeding on the dorsum of a coati. (**B**) Nymph of *Amblyomma sculptum* attached on the palate of a coati.

Regarding the first capture (used for statistical analyses), 455 larvae of *Amblyomma* spp. were obtained from both areas. The prevalence of infested coatis was 32/48 (66.67%) for PEP vs. 31/55 (56.36%) for VBA (**Figure 2**). For nymphs, a total of 735 specimens were collected, corresponding to 76 *A. dubitatum* and 659 *A. sculptum*. The prevalence of infested coatis with A. *dubitatum* was 23/48 (47.92%) for PEP vs. 21/55 (38.18%) for VBA; for *A. sculptum* it was 36/48 (75%) for PEP vs. 42/55 (76.36%) for VBA (Figure 2). Co-infestation by both *A. sculptum* and *A. dubitatum* nymphs was observed in 19 animals from PEP and 17 from VBA. Regarding adult ticks, only five specimens were sampled on four coatis during the first capture from VBA. One coati (VBA 27) was co-infested with one *A. sculptum* male and one *A. ovale* male. *Amblyomma ovale* adults were found only at VBA, while *A. sculptum* adults were found in both areas. Adult ticks were not included in the statistical analyses due to the small number of collected specimens.

3.3 Gender and Age

Taking into account the first capture, 43/63 (68.25%) females and 23/40 (57.5%) males were found parasitized by *Amblyomma* larvae (Figure 3). Considering that adult ticks were collected from only one female and three males, they were not included in the statistical analyses due to the small number of specimens collected. Taking into account the first capture, 12/26 (46%) immature and 51/77 (66%) mature animals were found parasitized by *Amblyomma* larvae (Figure 3). No adult tick was collected from immature

animals.

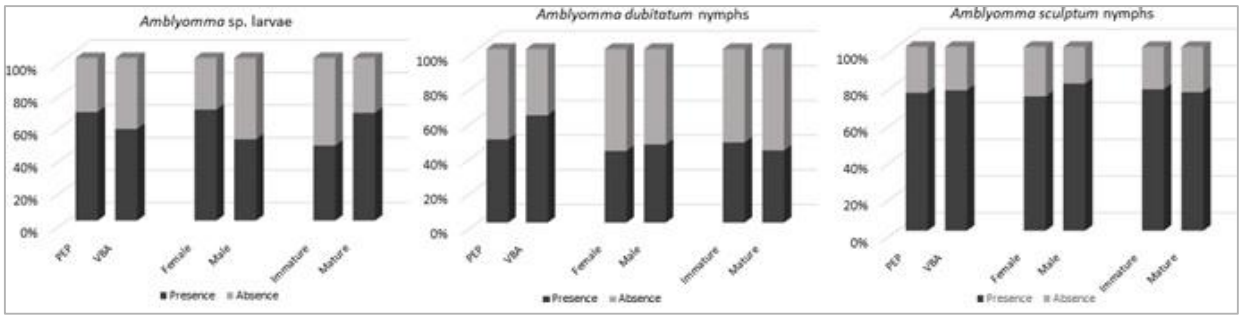

Figure 3. Graphical analyses demonstrating prevalence of coatis infested by *Amblyomma* larvae and nymphs of *A. dubitatum* and *A. sculptum* by percentage.

The proportion of coatis infested by *Amblyomma* larvae and nymphs of *A. dubitatum* and *A. sculptum* (evaluated separately) showed no significant statistical difference between the two sampling spots, gender, and age ($p > 0.05$). Values of confidence interval (superior and inferior), *Odds ratio* and *p*-value are showed in Table S1 (**Supplementary file**).

All descriptive values (total, intensity, and mean intensity) from non-recaptured animals are shown in Table 2. Effects of fixed factors (locality, gender, and age) and their interactions (locality and gender; locality and age, gender and age and locality, gender and age) were not observed on the quantity of ticks ($p > 0.05$—GLMM) (Supplementary file—Table S2). Therefore, we can assume that the prevalence (number of infested coatis ÷number of sampled coatis) and quantity of ticks (*Amblyomma* sp. larvae, *A. dubitatum* and *A. sculptum* evaluated separately) is not affected by age and gender of the coatis sampled in the present study and did not differ from PEP vs. VBA.

3.4 Seasonal Dynamics

Larvae of *Amblyomma* sp. and nymphs of both *A. dubitatum* and *A. sculptum* were sampled in all months of the year, but differences in the mean abundance of infestation were obtained. Regarding *Amblyomma* larvae, a higher mean of infestation was observed in the first months of the year (January, April and May). Nymphs presented opposite results, with higher mean of infestation in the second semester (July, August, October and November) when analyzing *A. sculptum* and *A. dubitatum* together. The thirteen adult ticks were collected in five different months (June 2018 —1 *A. ovale*, 1 *A. sculptum*), (August 2018—2 *A. ovale*, 2 *A. sculptum*, 1 *A. ovale*), (October 2018

—2 *A. sculptum*, 1 *A. ovale*) (November 2018 —1 *A. sculptum*), (January 2019 —1 *A. sculptum*), (March 2019—1 *A. sculptum*) (**Figure 4; Table 2**).

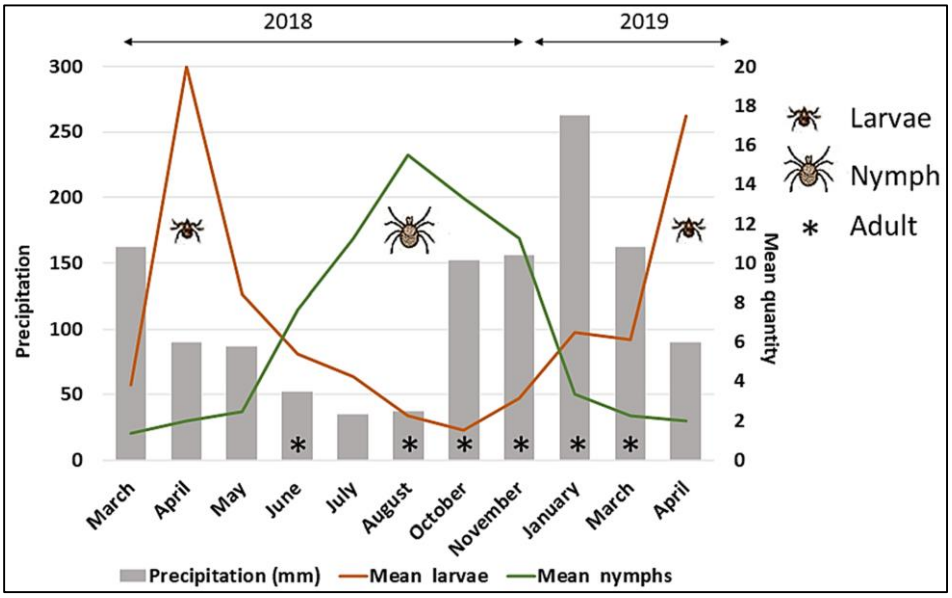

Figure 4. Mean quantity of tick larvae and nymphs collected from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil, according to the month of sampling, in the years of 2018 and 2019. Months when adult ticks were collected are showed as (*). Recaptures were included.

Table 2. Total, intensity, and mean intensity of tick specimens collected from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil, according to locality, gender and age. Recaptures were not included.

	Variables		*Amblyomma* spp. Larvae	*Amblyomma dubitatum* Nymphs	*Amblyomma sculptum* Nymphs	Adults
	Total in both areas		455 specimens	76 specimens	659 Specimens	5 specimens
PEP vs. VBA	Total by area	VBA [1]	277	46	384	5
		PEP [2]	178	30	275	-
	Intensity*	VBA	1–33	1–7	1–34	1–2 [+]
		PEP	1–31	1–4	1–23	-
	Mean intensity **	VBA	8.93 (277/31)	2.19 (46/21)	9.14 (384/42)	1.1 (11/10)
		PEP	5.56 (178/32)	1.3 (30/23)	7.63 (275/36)	-
	Total in both genders and ages		735 specimens	659 Specimens	5 specimens	5 specimens

Immature vs. Mature Female vs. Male	Total by gender	F[3]	286	42	458	1
		M[4]	169	34	201	4
	Total by age	IM[5]	102	24	134	-
		MAT[6]	353	52	225	5
	Intensity *	F	1–31	1–7	1–34	1[+]
		M	1 – 32	1–6	1–24	1 – 2
		IM	1 – 32	1–6	1–21	-
		MAT	1 – 31	1–7	1 - 34	1 – 2
	Mean intensity **	F	6.65 (286/43)	1.61 (42/26)	9.95 (458/46)	1 (1/1)
		M	8.45 (169/20)	1.88 (34/18)	6.48 (201/31)	1.33 (4/3)
		IM	8.5 (102/12)	2 (24/12)	6.07 (134/20)	-
		MAT	6.92 (353/51)	1.6 (52/32)	9.05 (525/58)	1.25 (5/4)

4. Discussion

Understanding the diversity and ecology of ectoparasites in wild animals is essential for surveillance of vector-borne diseases. Some changes in natural areas that lead to the absence of predators and the high availability of food can act as factors that lead to adaptation of coatis in anthropized environments, and thus the consequent increase in population densities [32]. Mainly in urban environments, the constant contact between wildlife, domestic animals and humans constitutes risk factors for the emergence of tick- borne diseases [44]. Also, some tick species that may be found in an anthropic environment may present a potential risk to public health, since they can transmit some important pathogens with zoonotic potential.

In the present study, three tick species were identified parasitizing coatis from mid-western Brazil, including *A. dubitatum* nymphs, *A. sculptum* adults and nymphs, and *A. ovale* adults. The dominant species, representing 89% of all collected nymphs was *A. sculptum*. A few specimens of adult *A. ovale* and nymphal *A. dubitatum* were also found, albeit in low numbers. Similar results were found when investigating ticks from wild carnivores (*Lycalopex vetulus, Cerdocyon thous, Chrysocyon brachyurus* and *Puma concolor*) in the Brazilian Cerrado, where *A. sculptum* represented 98.6% of the total number of sampled ticks, mainly in the nymphal stage [44]. The present study is also in agreement with other studies carried out in several regions of Brazil, which also indicated a strong association between *A. sculptum* nymphs and wild carnivores

[29,45,46]. *Amblyomma sculptum* is a generalist species, widely distributed in the Brazilian biomes of Cerrado, Pantanal, and degraded areas of the Atlantic forest [44,47–50]. Due to its adaptive plasticity, this species development is favored in degraded areas under anthropogenic influences [44,49]. In these degraded forest areas, *A. sculptum* is the most common human-parasitizing tick and has significant importance as the principal vector of the deadly Brazilian spotted fever pathogen, *Rickettsia rickettsii* [23,25,26]. Due to hunter and aggressive behavior of this tick species [23], the present report shows the importance of tick species monitoring, since the sampled coatis have access to places with high daily circulation of people (PEP) and close contact with human houses (VBA), making human bites and vector-borne pathogen transmission possible.

The present study includes the second report of *A. dubitatum* nymphs feeding on ring-tailed coatis. This tick species has already been found parasitizing coatis from São Paulo state [33]. All stages of *A. dubitatum* are primarily associated with capybaras (*H. hydrochaeris*), although there are reports of immature and adult stages in other mammals, such as tapirs (*Tapirus terrestris*) [51], crab-eating foxes (*C. thous*) [29], humans [23,52] as well as larvae and nymphs in black-eared opossums (*Didelphis aurita*) [53], white-eared opossums (*Didelphis albiventris*) [36,53], and Cricetidae rodents [54]. Interestingly, an interspecific association between coatis and capybaras was found in the Parque das Nações Unidas [55] located aside the PEP (where the present study was carried out). Rucco et al. [55] observed coatis feeding on ticks attached to capybaras, in a protocooperation between the two mammal species. Also, we observed capybaras resting aside from a coati captured in a trap in November 2021 at PEP (in a new campaign for a co-related study) (Figure S8). We suggest that the proximity of these two mammal species might have been associated with the presence of *A. dubitatum* nymphs on coatis; however, further studies are necessary to understand if this protocooperation can contribute to ticks' exchange between these two host species.

No statistically significant differences in tick species diversity, mean intensity, or prevalence was observed between coatis from the two sampled areas. PEP is a conserved area surrounded by urban environments and VBA is an anthropized environment surrounded by three forest fragments. Both areas have fluid movement of coatis between urban and wild areas, which may explain the lack of difference found in the present study. Biodiversity of host species in conserved areas, such as mammals, birds and reptiles, tend to be higher and more evenly distributed than in anthropized areas, since habitat loss and fragmentation may be a bias in the host species community, leading to the dominance of a few generalist species, such as coatis

[56,57]. Previous studies demonstrated that the abundance of some mammalian hosts has a direct effect on tick species/abundance, since the more the host abundance, the better the chances for ticks finding hosts to complete their life cycle, favoring the increase of a certain tick species population [56,58–60]. Although *A. sculptum* is primarily associated with capybaras, this tick species has also been described in a large variety of wild carnivores; indeed, this tick species is considered as a plastic species, adapting easily to anthropized environments. The fragmentation of both sampled areas that may serve to decrease the diversity of other mammal hosts might have led to the low diversity of tick species in the present study.

Interestingly, *A. ovale*, a tick species that is reported in wild carnivores and has already been described parasitizing coatis [29], was found only on coatis sampled in VBA. Unfortunately, no study on host diversity and abundance has been performed at PEP or VBA regarding the presence of wild carnivores, which in turn may help maintaining the *A. ovale* life cycle. Since we have no further information, it is impossible to assume that the occurrence of *A. ovale* in coatis only from VBA was due to a higher number of coatis sampled at VBA (which might have increased the possibility of collecting *A. ovale* adults); alternatively, VBA may have other wild carnivores along their forest fragments. Also, information is scarce regarding ticks parasitizing other hosts in the studied area. Previously, our research group [36] collected nymphs of A. *dubitatum* from *D. albiventris* at PEP.

Rhipicephalus (Boophilus) microplus and *Rhipicephalus sanguineus* sensu lato, both species related to domestic animals, were previously collected from free-living coatis in Pantanal, Mato Grosso do sul state [7], and captive coatis from a zoobothanical park from Paraíba state [33], respectively. *Rhipicephalus (Boophilus) microplus* has a worldwide distribution and is considered the most important tick of livestock in the world, due to the vector competence for *Babesia bigemina, Babesia bovis* and *Anaplasma marginale* [61,62]. Brazilian Pantanal is an important area for the cattle industry, where there are reports of wild animals sharing areas with cattle, which may lead to ticks exchange [63]. *Rhipicephalus sanguineus* is also found worldwide and is primarily associated with dogs, although there are reports of infestations of humans and other animals [64,65]. Both reports [7,33] found only one specimen of each *Rhipicephalus* species on coatis, which may be interpreted with caution, since it may be an occasional finding and therefore without epidemiological implications. Although both areas sampled in the present study have circulation of domestic animals (stray cats and dogs), no *R. sanguineus* or *R. (Boophilus) microplus* was collected from coatis. Understanding the parasite-host relationship is essential for investigating the dynamics of diseases and evolutionary implications of the

parasites in their hosts and ecosystems. A commonly observed pattern in this relationship is a higher intensity of pathogens in young animals compared to adult animals [66,67]. Reasons for this difference are not fully understood, and may vary according to the parasite species, host and environment. In the present study, no statistically significant difference was observed when comparing mean intensity and mean abundance of ticks between different ages (immature vs. mature). Taking into account the biology of the coati, it is known that they live in large groups, with cubs, subadults and adults sharing the same environment. Females give births in tree nests, and cubs leave those nests around five to eight weeks after birth [68,69], and then remain as subadults in the same group. We suggest that the absence of statistical differences may be due to the fact that coatis from all ages have similar chances to be in contact with arthropod vectors from the moment the cubs leave their nests. This type of observation may be important for future studies when comparing the chance of a coati from a certain age to be infected by a tick-borne pathogen.

The prevalence and intensity of parasitic infections appear to be higher in males than in females. This fact can be explained by the behavior of some species, in which males, which are more prone to aggressive interactions (to conquer females and territories) and dispersion, would be more likely to have contact with ectoparasites and associated pathogens [70]. However, several studies have found no gender differences in the prevalence or intensity of parasite infection [33,71], and some have even found higher rates of parasite infection in females [72]. No statistically significant differences were observed between genders of coatis. Previous studies suggested that *N. nasua* would have a similar social system to *Nasua narica*. While solitary adult males live apart from the group outside the mating season, females live in groups with their offspring [73–75]. This behavior may lead to higher prevalence and intensity of ectoparasites and parasitic infections in males than in females because they tend to disperse in a larger territory [70]. However, studies about dietary patterns and behavior of *N. nasua* showed different features, with adult males observed living together with females and offspring outside of the mating season in five different coati populations in Brazil (Mangabeiras Park, Minas Gerais; Tiete Ecological Park, São Paulo; Nhumirim ranch, Pantanal; *Parque Estadual do Prosa*, Mato Grosso do Sul; and Campeche Island, Santa Catarina) and in Foz do Iguaçu, Argentina, [1,13,34–36]. It seems that both in natural [69] and anthropized areas (such as those sampled in the present study) males remain together with the group outside of the mating season. This behavior might explain the lack of statistical differences in the presence of ticks between males and females, since they cohabit the same environment.

Conclusions

Three tick species were identified parasitizing coatis from midwestern Brazil, namely *A. dubitatum* nymphs, *A. sculptum* adults and nymphs, and *A. ovale* adults. We observed low tick species diversity on coatis from two highly anthropized areas. The lack of statistical difference regarding mean intensity and prevalence of *Amblyomma* larvae and nymphs between males and females and immature and mature animals might have reflected the gregarious behavior of coatis, since adult males live together with females and offspring outside and inside the mating season, forming large groups of individuals.

Supplementary Materials: The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/ani12030293/s1, Figure S1: Non-engorged *Amblyomma* spp. larvae collected from a ring-tailed coati (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. Figure S2: *Amblyomma sculptum* nymphs collected from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. Figure S3: *Amblyomma dubitatum* nymphs collected from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. Figure S4: *Amblyomma ovale* male collected from a ring-tailed coati (*Nasua nasua*) in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. Figure S5: *Amblyomma ovale* engorged female collected from a ring-tailed coati (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. Figure S6: *Amblyomma sculptum* male collected from a ring-tailed coati (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. Figure S7: *Amblyomma sculptum* non- engorged female sampled in a ring-tailed coati sampled (*Nasua nasua*) in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. Figure S8: Photographic image of a capybara (*Hydrochoerus hydrochaeris*) (red arrow) near a coati (*Nasua nasua*) in a trap (yellow arrow). Table S1: Values of Odds ratio, Confidence interval (inferior and superior) and *p*-value obtained in the analysis on prevalence of ticks on coatis (*Nasua nasua*) sampled from March 2018 to April 2019 in Campo Grande city, Mato Grosso do sul state, Brazil. Table S2: *p*-value obtained in the analysis on quantity of ticks (*Amblyomma* sp. larvae, *Amblyomma dubitatum* and *Amblyomma sculptum* nymphs) and the interactions between variables locality, sex and age.

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Data Availability Statement: The raw data generated in this study can be obtained by reasonable request to the corresponding author.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

Supplementary file. Voucher material deposited in the IBSP collection.

***Amblyomma* sp.**

10 larvae (IBSP 16757); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 02 Aug. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll.;5 larvae (IBSP 16774); same locality; same host; 11 May 2018; 4 larvae (IBSP 16802A); 30 May. 2018; 6 larvae (IBSP 16806A); same data; 4 larvae (IBSP 16779); 06 Aug. 2018; 2 larvae (IBSP 16801); 13 Mar. 2018; 1 larva (IBSP 16805); same day; 4 larvae (IBSP 16807B); same locality; same host; 31 Jul. 2018; 8 larvae (IBSP 16809B); 15 Jun. 2018; 2 larvae (IBSP 168010B); 30 May 2018; 7 larvae (IBSP 16812A); 15 Mar. 2018; 1 larva (IBSP 16813); 16 Mar. 2018; 1 larva (IBSP 16814A); 16 Mar. 2018; 1 larva (IBSP 16815B); same data; 3 larvae (IBSP 16816A); 19 Mar. 2018; 3 larvae (IBSP 16816A); 19 Mar. 2018; 1 larva (IBSP 16817B); 20 Mar. 2018; 3 larvae (IBSP 16818A); 21 Mar. 2018; 16 larvae (IBSP 16821B); 15. May 2018; 4 larvae (IBSP 16823A); same data; 31 larvae (IBSP 16822B); 22. May 2018; 5 larvae (IBSP 16824); 28. May 2018; 3 larvae (IBSP 16825); same data; 5 larvae (IBSP 16826); 29 May 2018; 3 larvae (IBSP 16827A); same data; 4 larvae (IBSP 16828B); same data; 18 larvae (IBSP 16804A); 30 May. 2019; 7 larvae (IBSP 16829B); 30 May 2018; 19 larvae (IBSP 16830); 12 Jun. 2018; 9 larvae (IBSP 16831); 12 Jun. 2018; 3 larvae (IBSP 16834A); 31 Jul. 2018; 5 larvae (IBSP 16835C); same data; 3 larvae (IBSP 16836A); 01 Aug. 2018; 16 larvae (IBSP 16837A); 02 Aug. 2018; 4 larvae (IBSP 16839A); 03 Aug. 2018; 9 larvae (IBSP 16840B); same data; 3 larvae (IBSP 16842A); 06 Aug. 2018; 4 larvae (IBSP 16844); 08 Aug. 2018; 13 larvae (IBSP 16765); same host; Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 23 March 2019; 3 larvae (IBSP 16766B); same host; same locality; 01 Nov. 2018; 15 larvae (IBSP 16767B); same host; same locality; 30 Jan. 2019; 20 larvae (IBSP 16768); same host; same locality; 29 Jan. 2019; 1 larva (IBSP 16771); 26 Jun. 2018; 7 larvae (IBSP 16772); 29 Jan. 2019; 1 larva (IBSP 16773); 23 Jan. 2019; 33 larvae (IBSP 16777); 30 Apr. 2019; 33 larvae (IBSP 16779B); 06 Aug. 2018; 7 larvae (IBSP 16782); 18 May 2018; 1 larva (IBSP 16785A); 24 Aug 2018; 2 larvae (IBSP 16788); 20 May 2018; 1 larva (IBSP 16793); 21 Mar. 2019; 32 larva (IBSP 16797); 25 Mar. 2019; 20 larva (IBSP 16847A); 30 Apr. 2018; 11 larva (IBSP 16848); 02 May. 2018; 3 larvae (IBSP 16850B); 28 Aug. 2018; 15 larvae (IBSP 16852A); 02 May 2018; 2 larvae (IBSP 16855); same data; 5 larvae (IBSP 16856); 03 May 2018.

***Amblyomma ovale* (Koch 1844)**

1 ♀ (IBSP 16854); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 21 Aug. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 ♀ (IBSP 16860B); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 19 May. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 ♀ (IBSP 16863C); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 04 May. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora:

Procyonidae); L. Perles et al. coll. 1 ♂ (IBSP 16862); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 23 Oct. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 ♂ (IBSP 16769A); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 20 Aug. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 ♀ (IBSP 16864); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 04 May 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae).

Amblyomma dubitatum **(Neumann 1899)**

1 nymphae (IBSP 16749); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 23 Jan. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 5 nymphs (IBSP 16750); same data; 2 nymphae (IBSP 16754); same locality and host; 30 April 2019; 1 nymphae (IBSP 16758); same locality; same host; 22 Jan. 2019; 1 nymphae (IBSP 16759A); same locality; same host; 06 Nov. 2018; 1 nymphae (IBSP 16764); same locality; same host; 30 Aug. 2018; 1 nymph (IBSP 16767A); same locality; same host; 30 Jan. 2019; 1 nymphae (IBSP 16770); same locality; same host; 23 Apr. 2019; 1 nymphae (IBSP 16776); same locality; 29 Jan. 2019; same host; 1 nymphae (IBSP 16780B); same locality; 23 Jan. 2019; 1 nymphae (IBSP 16786); 30 Jan. 2019; 1 nymphae (IBSP 16787); 31 Out. 2018; 1 nymphae (IBSP 16798); same locality; same host; 24 Aug. 2018; 1 nymphae (IBSP 16753B); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 04 Out. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16800); 31 July 2018; 1 nymphae (IBSP 16803B); 13 Mar. 2018; 1 nymphae (IBSP 16806C); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 30 May. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16808B); 14 Mar. 2018; 1 nymphae (IBSP 16812B); 15 Mar. 2018; 1 nymphae (IBSP 16816B); 19 Mar. 2018; 1 nymphae (IBSP 16818B); 21 Mar. 2018; 1 nymphae (IBSP 16819); 21 Mar. 2018; 1 nymphae (IBSP 16827B); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 29 May. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16832A); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 31 Jul. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16833A); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 31 Jul. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16835A); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 31 Jul. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16836B); 01 Aug. 2018; 1 nymphae (IBSP 16838A); 02 Aug. 2018; 1 nymphae (IBSP 16839B); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 03 Aug. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16842B); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 06 Aug. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymph (IBSP 16853C); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 02 May. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16863B); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 04 May. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16878A); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 24 Aug. 2018; 1 nymphae (IBSP 16881); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul

State, Brazil; 31 Out. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll. 1 nymphae (IBSP 16882); 31 Aug. 2018.

Amblyomma sculptum (Berlese 1888)

4 nymphae (IBSP 16802B); Parque Estadual do Prosa, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; 30 May. 2018; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); L. Perles et al. coll.; 7 nymphae (IBSP 16804B); same data; 18 nymphae (IBSP 16792); same locality; same host; 8 Oct. 2018; 1 nymph (IBSP 16803A); 13 Mar. 2018; 4 nymphae (IBSP 16806B); same locality; same host; 30 May 2018; 9 nymphae (IBSP 16807A); same locality; same host; 31 Jul. 2018; 1 nymph (IBSP 16808A); same locality; same host; 14 Mar. 2018; 3 nymphae (IBSP 16809A); same locality; same host; 15 Jun. 2018; 9 nymphae (IBSP 16790); same locality; same host; 30 Jul. 2018; 10 nymphae (IBSP 16810A); same locality; same host; 30 May 2018; 11 nymphae (IBSP 16755); same locality; same host; 1 Aug. 2018; 1 nymph (IBSP 16814B); same locality; same host; 16 Mar. 2018; 2 nymphae (IBSP 16815A); same data; 1 nymph (IBSP 16817A); same locality; same host; 20 Mar. 2018; 2 nymphae (IBSP 16818C); same locality; same host; 21 Mar. 2018; 2 nymphae (IBSP 16820B); same locality; same host; 22 Mar. 2018; 4 nymphae (IBSP 16822A); same data; 2 nymphae (IBSP 16821A); same locality; same host; 15 Jun. 2018; 7 nymphae (IBSP 16823B); same data; 17 nymphae (IBSP 16762); same locality; same host; 1 Oct. 2018; 1 nymph (IBSP 16828A); same locality; same host; 29 May 2018; 12 nymphae (IBSP 16829A); same locality; same host; 30 May 2018; 30 nymphae (IBSP 16781); same locality; same host; 30 Jul. 2018; 8 nymphae (IBSP 16832B); same locality; same host; 31 Jul. 2018; 6 nymphae (IBSP 16833B); same data; 4 nymphae (IBSP 16834B); same data; 7 nymphae (IBSP 16835B); same data; 7 nymphae (IBSP 16836C); same locality; same host; 01 Aug. 2018; 6 nymphae (IBSP 16837B); same locality; same host; 02 Aug. 2018; 8 nymphae (IBSP 16838B); same data; 11 nymphae (IBSP 16799); same locality; same host; 04 Oct. 2018; 10 nymphae (IBSP 16839C); same locality; same host; 31 Jul. 2018; 11 nymphae (IBSP 16840); same locality; same host; 03 Aug. 2018; 17 nymphae (IBSP 16841); same locality; same host; 03 Aug. 2018; 15 nymphae (IBSP 16779A); same locality; same host; 06 Aug. 2018; 7 nymphae (IBSP 16843); same locality; same host; 07 Aug. 2018; 4 nymphae (IBSP 16844); same locality; same host; 08 Aug. 2018; 17 nymphae (IBSP 16845); same data; 2 nymphae (IBSP 16847B); Vila da Base Aérea, Campo Grande Municipality, Mato Grosso do Sul State, Brazil; ex. *Nasua nasua* (Linnaeus 1766) (Carnivora: Procyonidae); 30 Apr. 2018; L. Perles et al. coll. 7 nymphae (IBSP 16849); same locality; same host; 18 Jun. 2018; 9 nymphae (IBSP 16851); same locality; same host; 23 Oct. 2018; 2 nymphae (IBSP 16852B); same locality; same host; 1 nymph (IBSP 16853B); same data; 19 nymphae (IBSP 16761); same locality; same host; 22 Oct. 2018; 8 nymphae (IBSP 16860A); same locality; same host; 19 Jun. 2018; 1 nymph (IBSP 16861B); same locality; same host; 4 May 2018; 1 nymph (IBSP 16863B); same data; 1 nymph (IBSP 16867A); same locality; same host; 10 May 2018; 2 nymphae (IBSP 16847B); same locality; same host; 33 nymphae (IBSP 16752); same locality; same host; 31 Aug. 2018; 27 nymphae (IBSP 16785B); same locality; same host; 24 Aug. 2018; 4 nymphae (IBSP 16869B); same locality; same host; 21 Jun. 2018; 4 nymphae (IBSP 16870); same locality; same host; 22 Jun. 2018; 13 nymphae (IBSP 16871); same locality; same host; 22 Jun. 2018; 12 nymphae (IBSP 16872B); same locality; same host; 24 Aug. 2018; 1 nymph (IBSP16873); same locality; same host; 21 Mar. 2018; 11 nymphae (IBSP 16875); same locality; samehost; 26 Jun. 2018; 9 nymphae (IBSP 16875B); same locality; same host; 26 Jun. 2018; 14 nymphae (IBSP 16760); same locality; same host; 06 Nov. 2018.

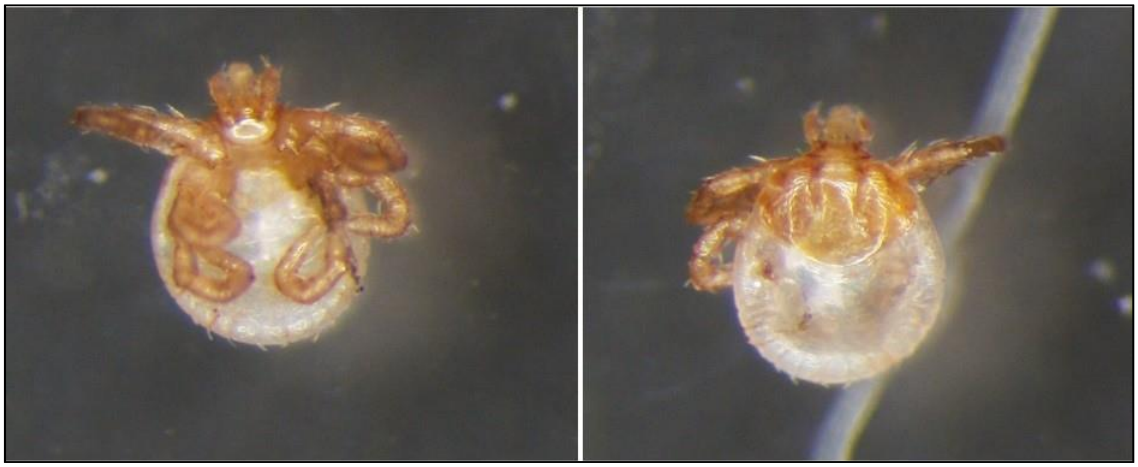

Figure S1. Non-engorged *Amblyomma* spp. larvae collected from a ring-tailed coati (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. A) Ventral view. B) Dorsal view.

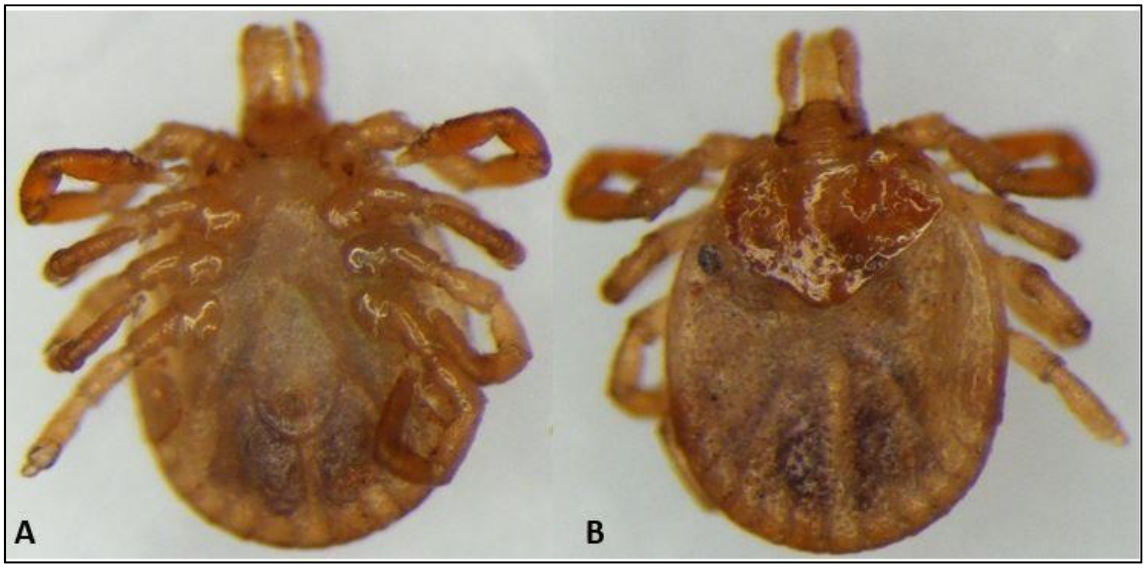

Figure S2. *Amblyomma sculptum* nymphs collected from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. A) Ventral view. B) Dorsal view. GenBank acession number: MT974144.

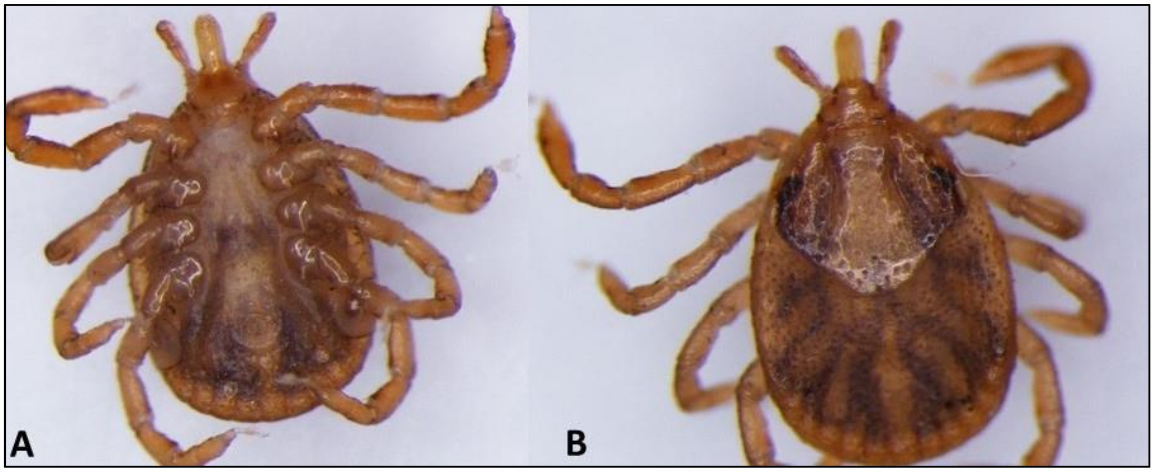

Figure S3. *Amblyomma dubitatum* nymphs collected from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. A) Ventral view. B) Dorsal view. GenBank acession number: MT974145.

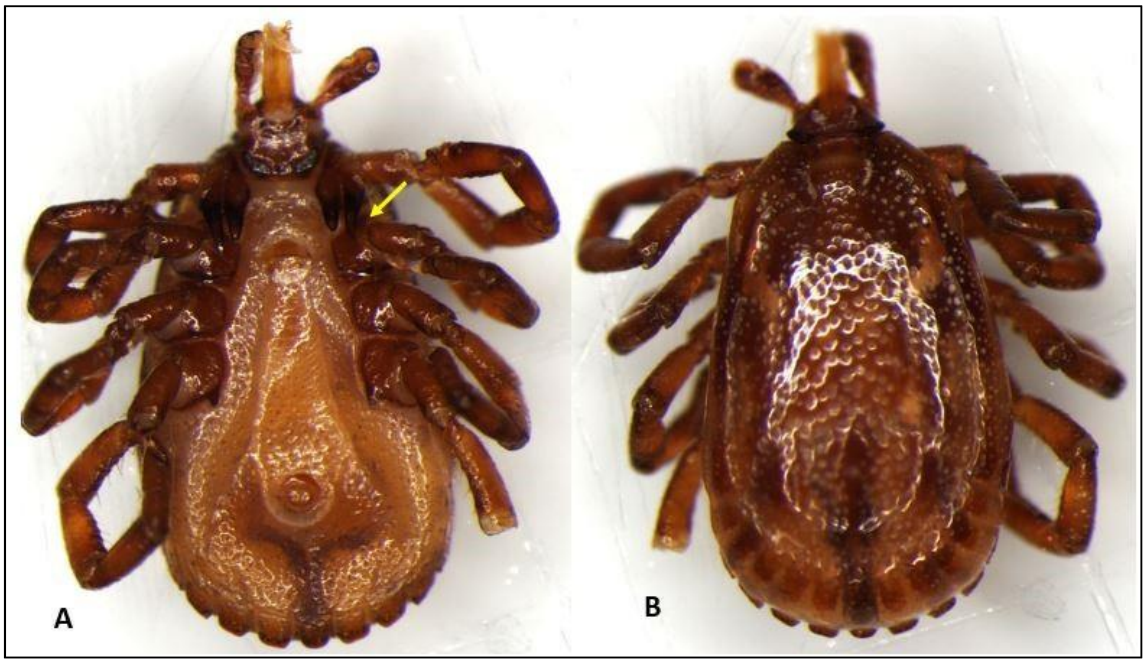

Figure S4. *Amblyomma ovale* male collected from a ring-tailed coati (*Nasua nasua*) in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. A) Ventral view: two strong spines can be seen on thigh 1, with the outside spine turned slightly outwards (arrow). B) Dorsal view.

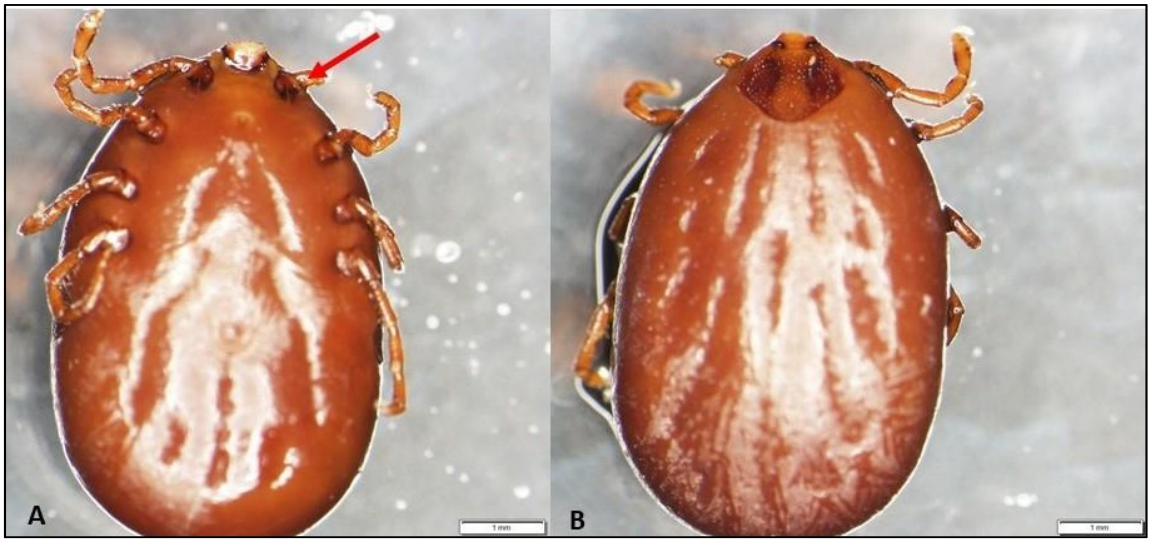

Figure S5. *Amblyomma ovale* engorged female collected from a ring-tailed coati (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. A) Ventral view: two strong spines can be seen on thigh 1, with the outside spine turned slightly outwards (arrow). B) Dorsal view.

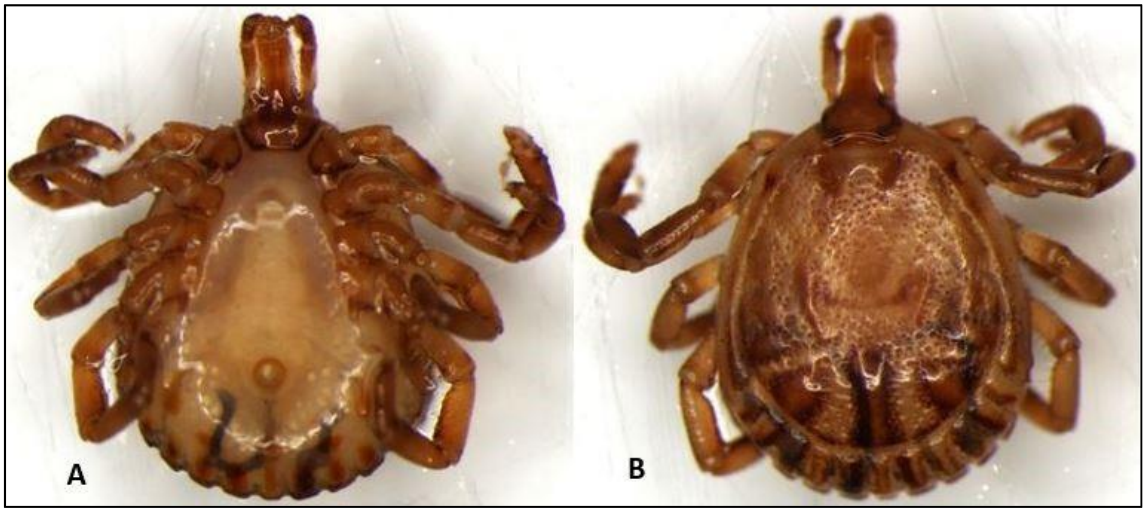

Figure S6. *Amblyomma sculptum* male collected from a ring-tailed coati (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. A) Ventral view. B) Dorsal view.

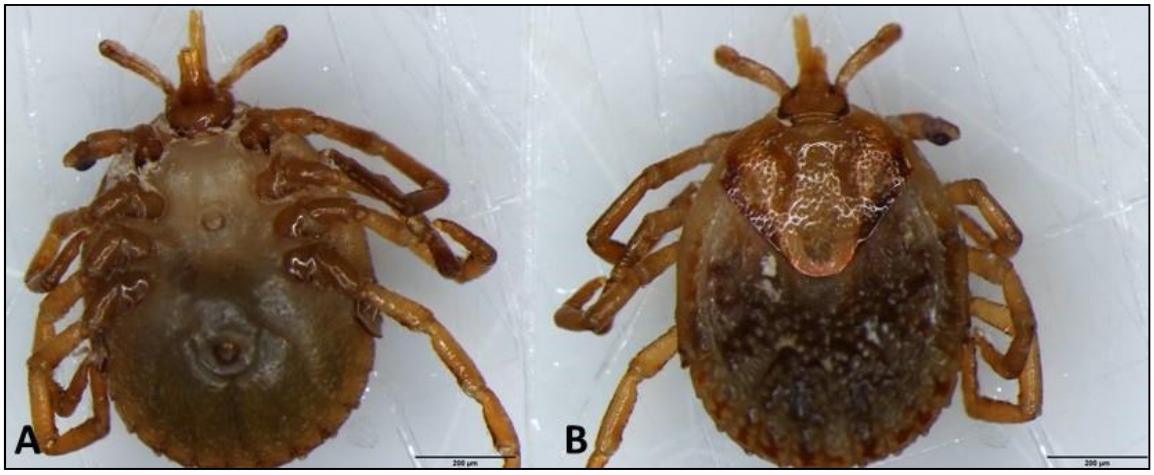

Figure S7. *Amblyomma sculptum* non- engorged female sampled in a ring-tailed coati sampled (*Nasua nasua*) in Campo Grande city, Mato Grosso do Sul state, central-western Brazil. A)Ventral view. B) Dorsal view.

Figure S8. Photographic image of a capybara (*Hydrochoerus hydrochaeris*) (red arrow) near acoati (*Nasua nasua)* in a trap (yellow arrow).

Table S1. Values of Odds ratio, Confidence interval (inferior and superior) and *p*-valueobtained in the analysis on prevalence of ticks on coatis (*Nasua nasua*) sampled from March 2018 to April 2019 in Campo Grande city, MatoGrosso do sul state, Brazil.

Table S2. *P*-value obtained in the analysis on quantity of ticks (*Amblyomma* sp. larvae, *Amblyomma dubitatum* and *Amblyomma sculptum* nymphs) and the interactions between variables locality, sex and age.

	p-value		
Variable	*Amblyomma* sp. larvae	*Amblyomma dubitatum* nymphs	*Amblyomma sculptum* nymphs
Locality	0.29	0.21	0.35
Sex	0.94	0.83	0.76
Locality and sex	0.47	0.62	0.83
Age	0.51	0.97	0.30
Locality and age	0.77	0.17	0.40
Sex and age	0.63	0.46	0.21
Locality sex and age	0.74	0.97	0.48

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Capítulo 3* - Contribution to the knowledge of *Neotrichodectes (Nasuicola) pallidus* (Piaget, 1880) (Phthiraptera: Trichodectidae)

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Abstract

The species in the genus *Neotrichodectes* (Phthiraptera: Ischnocera) infest carnivores. *Neotrichodectes* (*Nasuicola*) *pallidus* (Piaget, 1880), which has been primarily found parasitizing Procyonidae mammals, has been recorded in ring-tailed coatis (*Nasua nasua*) in the Brazilian states of Minas Gerais, Pernambuco, Santa Catarina, Rio Grande do Sul and Pernambuco. We report a new record of *N. pallidus* in coatis in the state of Mato Grosso do Sul, central-western Brazil, using morphological (Light and Scanning Electronic Microscopy) and molecular approaches (PCR, sequencing and phylogenetic analysis). Coatis were sampled in two peri-urban areas of Campo Grande city, Mato Grosso do Sul state, Brazil, between March 2018 and March 2019, as well as in November 2021. Lice were collected and examined under light and Scanning Electron Microscopy. DNA was also extracted from nymphs and adults and submitted to PCR assays based on the 18S rRNA and *cox-1* genes for molecular characterization. One hundred and one coatis were sampled from 2018-2019 and 20 coatis in 2021 [when the intensity of infestation (II) was not accessed]. Twenty-six coatis (26/101 – 25.7%) were infested with at least one louse, with a total of 59 lice collected in 2018-2019. The II ranged from one to seven lice (mean 2.2 ± SD 1.7). The louse species was confirmed based on the following morphological characteristics: female gonapophyses rounded with the setae along anterior region but not in the medial margin; the male genitalia with a parameral arch not extending beyond the endometrial plate. The same ornamentation was observed on the abdomen of the females, males, and nymphs. The nymphs and the eggs were described in detail for the first time. The

obtained 18S rRNA and *cox1* sequences from *N. pallidus* clustered in a clade with other sequences of Ischnocera species. In the present study, a new record of the louse N. pallidus in central-western Brazil was provided, along with new insights into the morphological features of this species, with the first morphology contribution of nymphal and eggs stages.

Key-Words: louse, *Nasua nasua*, 18S rRNA, *cox-1*, phylogeny, SEM

Introduction

The Parvorder Phthiraptera (Order Psocodea) comprises approximately 5,000 species that parasitize birds and mammals, which consists of four suborders, namely Ischnocera, Amblycera, Anoplura and Rhyncophthirina (Johnson et al., 2003; Galloway, 2018; Durden, 2019). The louse species belonging to the suborders Ischnocera and Amblycera, occur in the Neotropical region and are popularly known as chewing lice, since their diet is mainly composed by necrotic epidermal fragments, hair, feathers and sebaceous secretions from their hosts and blood in some species (Urquhart et al., 1987; Boyd, Reed 2012; Galloway, 2018; Durden, 2019). Although an infestation by chewing lice is not usually considered harmful to the host, lice can cause lesions on the skin and, consequently, open entryways for many pathogens (Marcondes, Linardi 2017; Benelli et al., 2018). Indeed, some species can cause stress and weight loss to the host (e.g. *Menacanthus stramineus* (Nitzsch, 1818) infesting chicken) (Paliy et al., 2018; Kouam et al., 2022).

Within the Ischnocera, the family Trichodectidae Kellogg, 1896 comprises species associated with domestic and wild animals, such as *Felicola subrostratus* (Burmeister, 1838) and *Trichodectes canis* (De Geer, 1818) that parasitize domestic cats and dogs, respectively. *Felicola subrostratus* and *T. canis* are intermediate hosts for the zoonotic tapeworm *Dipylidium caninum* (Rousseau et al., 2022). Particularly, *Neotrichodectes*, has been associated with species of the order Carnivora. *Neotrichodectes* (*Nasuicola*) *pallidus* (Piaget, 1880) is known to infest only species of the *Nasua* (Price et al. 2003). In South America, this species infests ring-tailed coatis, *Nasua nasua* (L., 1766) in the Brazilian states of Minas Gerais (Rodrigues et al., 2006; Estevam et al., 2020), Paraná (Magalhães-Matos et al., 2017), Pernambuco (Vale, 2020), Rio Grande do Sul, and Santa Catarina (Picolli, 2010), and in Venezuela (Emerson, Price 1975; Canizales, Guerrero 2017), Bolivia, Colombia, and Paraguay (Werneck 1948). Additionally, this louse species has been recorded in Latin America, including Mexico (Werneck 1948; Sánchez-Montes et al., 2018; Guzman-Cornejo et al., 2020), Panama, (Wernek 1948), and Nicaragua (Emerson, 1971) where it infests white-nosed coati, *Nasua narica* (L., 1766) (Carnivora: Procyonidae).

In the present study, we report a new Brazilian locality for *N. pallidus* associated with the ring-tailed coati. Additionally, we present the first molecular data for this species of louse and the first detailed morphological images using scanning electron microscopy.

Material and Methods

Sampling of lice

All captures were performed in two Conservation areas, “Parque Estadual do Prosa” (PEP) (-20.44987, -54.56529) and “Vila da Base Aérea” (VBA) (-20.47163, -54.65405), both located in Campo Grande city, Mato Grosso do Sul state, in midwestern Brazil. PEP is a state National Conservation Park 134 ha in size, representing one of the last remnants of the Cerrado biome within the urban perimeter. The area has become a tourist spot for visitors from cities in the state of Mato Grosso do Sul as well as from different regions of Brazil, so there is a high daily circulation of people. VBA is a residential area, surrounded by three Cerrado forest fragments and one area used for military training, with approximately 484 ha. At least 730 people and their domestic animals inhabited the residential complex.

Ring-tailed coatis were captured every three weeks for 10 consecutive days between March of 2018 and April of 2019. Animals were captured using metal traps (1m x 0.40m x 0.50m) placed arbitrarily according to the possibility of human access and availability of shadow, covering most of the PEP and VBA areas. Animals were anesthetized with an association of Tiletamine hydrochloride and Zolazepam hydrochloride (Telazol, Zoetis® (Parsippany-Troy Hills, NJ, USA) (5-7 mg/kg, intramuscularly). After chemical restraint, animals were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue between the shoulder blades in order to identify them for future recaptures. Animals were measured and the age was estimated according to Olifiers et al. (2010). The animals’ entire body was inspected for the presence of louse for three minutes. Louse were removed with the aid of a forceps and stored in 100% alcohol (Merck Ensure®, Darmstadt, Germany)- containing RNAse/DNAse free microtubes. Taxonomic identification of ectoparasites was performed with a stereoscopic microscope (Olympus SZX7, Tokyo, Japan). Overall fur/skin condition and weight of the animals were assessed. The infestation intensity corresponded to the number of lice collected from each animal. In November 2021, a new sampling procedure was performed, but the infestation intensity was not accessed. Such samplings have already resulted in several other publications about tick-borne agents in coatis and ecological features of the species in urban areas of Central-Western Brazil (Barreto et al., 2021; Perles et al., 2022a, b, c). However, the assessment of infestation by lice on these animals has yet to be addressed.

Morphological analysis

Louse specimens were studied using three methods: (1) slide-mounting for identification by light microscopy, (2) preparation and observation using scanning electron microscopy (SEM), and (3) molecular characterization. Lice were identified using previously described taxonomic keys (Werneck 1936; Price et al. 2003) and all the original descriptions for the species into this genus. All these preparations were made based on Barros-Battesti et al. (2021). The material was deposited at the Entomological Collection of the *Instituto Butantan*, São Paulo, Brazil (IBSP - Ent).

The images were obtained using Leica DFC 500 digital camera coupled to a Leica DM4000B light microscope. Extended focal range images were composed using Leica Application Suite version 2.5.0. The SEM images were obtained with a Digital Scanning Microscope FEI, Quanta 250, located at the *Laboratório de Biologia Celular, Instituto Butantan*, São Paulo, SP, Brazil (IBSP).

DNA extraction and molecular characterization

Five representatives of each stage (female, male and nymph) obtained during the sampling performed between 2018 and 2019 were selected randomly and submitted individually to DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen®), according to manufacturers' instructions. Vouchers were preserved to confirm the identification and corroborate the morphological results. Conventional PCR assays targeting fragments of the 18S rRNA and *cox1* genes were performed following previously described protocols (Folmer et al. 1994; Otto & Wilson 2001). Amplicons were purified using Exosap (Life Technologies®, Carlsbad, CA, USA) and subjected to sequencing by the Sanger method using ABI PRISM 3730 DNA Analyzer (Applied Biosystems) at the Human Genome and Stem Cell Research Center, *Instituto de Biociências*, University of São Paulo (USP), São Paulo, SP, Brazil. Due to economic limitations, only one sequence from each life stage (male, female and nymph) was sequenced for *cox*1 gene. Using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), we could determine the Query-coverage %, E-value, and % identity of the obtained sequences. The phylogenetic analyses were based on Bayesian inference (BI) and performed using MrBayes version 3.1.2 (Ronquist, Huelsenbeck 2003) via the CIPRES Science Gateway. The phylogenetic tree edition and rooting (outgroup) were performed using TreeGraph 2.0 beta software (Stover, Muller 2010).

Results

In total, 101 coatis where sampled [51 in PEP (30 females and 21 males) and 60 in VBA (34 females and 26 males)] during 2018-2019. Twenty-six coatis [26/101 – 25.7% (13 females and 13 males)] were infested with at least one louse, with a total of 59 lice obtained (27 nymphs, 15 females and 17 males; see **Table 1**). The intensity of infestation ranged from 1 to 7 lice (mean 2.2 ± SD 1.7). From PEP, 10 (19.6%) animals were infested [three females (IR: one - two lice) and seven males (IR: one – four)]. From VBA, 16 (26.6%) animals were infested [10 females (IR: one – five) and six males (IR: one – seven).

Tables

Table 1. Identification of South American coatis (*Nasua nasua*) infested with *Neotrichodectes* (*Nasuicola*) *pallidus* sampled in Campo Grande city, Mato Grosso do Sul state, Brazil, during 2018 and 2019. Information is shown about sample localities , date of collection, sex of the hosts, intensity of infestation and life stages of lice collected.

Identification	Localization	Sampling data	Sex	Infestation rate	Stages
ID04	PEP[1]	14/03/2018	M[3]	4	1F, 3N[5]
ID08	PEP	15/03/2018	F[4]	1	1N
ID09	PEP	16/03/2018	F	1	1M
ID10	PEP	16/03/2018	M	2	1F, 1M
ID11	PEP	16/03/2018	F	2	1N, 1F
ID13	PEP	20/03/2018	M	1	1M
ID88	PEP	07/08/2018	M	2	2F
ID93	PEP	09/08/2018	M	1	1M
ID121	PEP	02/10/2018	M	1	1N
ID123	PEP	02/10/2018	M	1	1N
ID48	VBA[2]	18/06/2018	F	2	1N, 1M
ID155	VBA	23/01/2019	F	2	2N

ID165	VBA	20/03/2019	F	1	1N
ID98	VBA	21/08/2018	M	3	1N, 2F
ID61	VBA	25/06/2018	M	1	1N
ID104	VBA	27/08/2018	F	4	2F, 2M
ID105	VBA	27/08/2018	F	1	1N
ID107	VBA	28/08/2018	F	5	1N, 2F, 2M
ID109	VBA	28/08/2018	M	1	1N
ID110	VBA	28/08/2018	M	1	1N
ID112	VBA	29/08/2018	F	4	1F, 3M
ID153	VBA	23/01/2019	F	1	1F
ID154	VBA	23/01/2019	F	1	1F
ID161	VBA	29/01/2019	M	3	2N, 1M
ID167	VBA	21/03/2019	F	6	3N, 3M
ID169	VBA	21/03/2019	M	7	5N, 1F, 1M
			Total	59	27N, 15F, 17M

[1] Parque Estadual do Prosa; [2] Vila da Base Aérea; [3] Male; [4]Female; [5]Nymph

Regarding weight, all parasitized animals presented good body condition, and weights were within the normal range for the species. Regarding overall skin/full conditions, 10 animals showed skin lesions (4 from PEP and 6 from VBA) consisting of scars in different body regions (nose, legs,), suggestive of aggressive interactions but not related to the presence of lice.

In November 2021, twenty animals were sampled (the infestation intensity was not accessed). All collected specimens (nymphs and adults) we identified to species level. At this moment, louse eggs were also sampled.

After slide-mounting the chewing louse specimens sampled, the species was identified as *N. pallidus* based on the following set of morphological characteristics: female gonapophyses rounded with the setae along the anterior region but not in the medial margin (**Figure 1**); male genitalia with a parameral arch not extending beyond the endometrial plate (**Figure 2**). Furthermore, for the entire genus, the spiracles are absent in the abdomen; the endometrial plate and parameral arch have an apical pointed process in the male genitalia. For the first time, the nymphs and the eggs of this chewing louse species (**Figure 3A-C** and **3D, respectively**) were photographed and their details recorded. The elliptical eggs have a protective cover in the anterior region, with subtle small balls adorning the entire surface (Figure 3D). This ornamentation resembles a scale-like ornamentation that the females (**Figure 1A**), males (**2A**), and nymphs (**Figure 3A**) have.

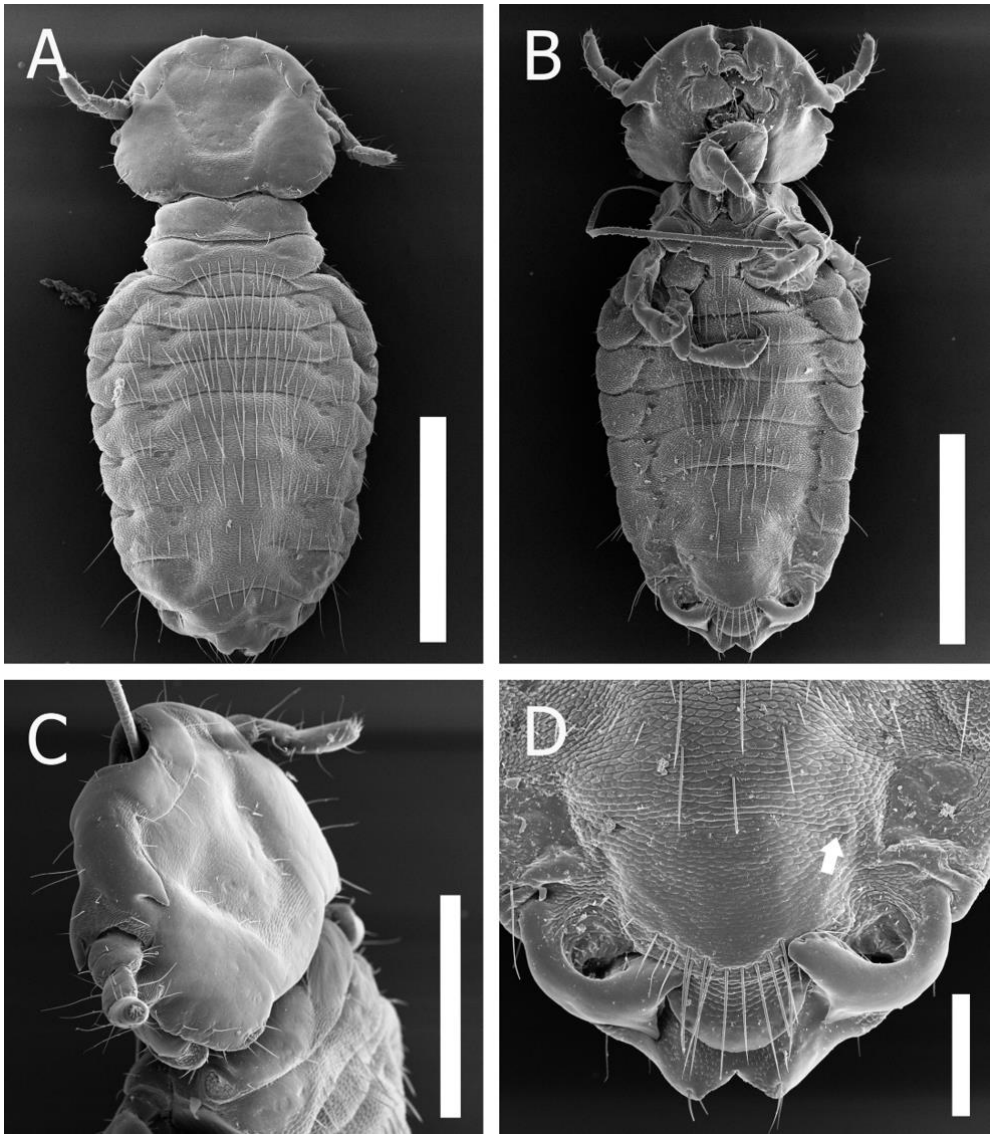

Figure 1. SEM images of the *Neotrichodectes* (*Nasuicola*) *pallidus* (Piaget, 1880) female. **A** – dorsal; **B** – ventral; **C**- head; **D** –distal part of the ventral region. White arrow highlights the scale-like ornamentation. Scale bars: **A** and **B** 500 μm, **C** 300 μm and **D** 100 μm.

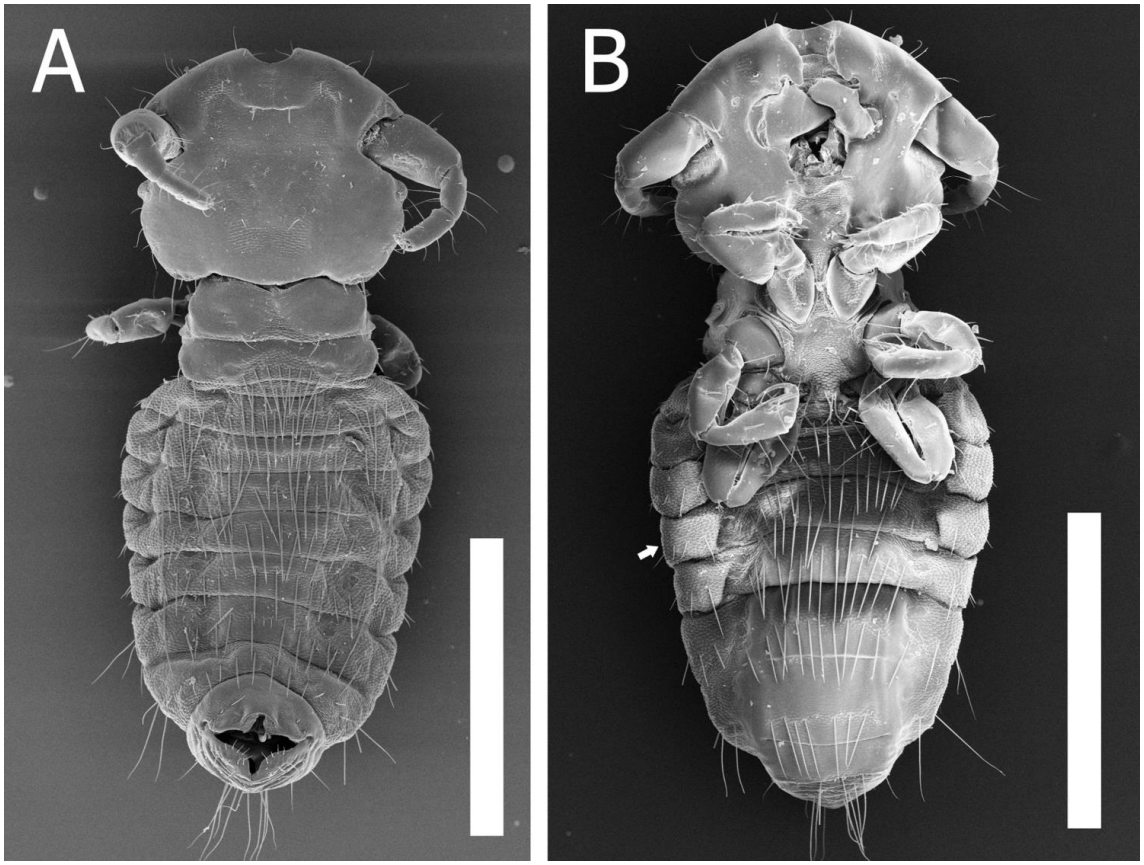

Figure 2. SEM images of the *Neotrichodectes* (*Nasuicola*) *pallidus* (Piaget, 1880) male. **A** – dorsal; **B** – ventral. White arrow highlights the absence of spiracles. Scale bars: **A** and **B** 500 μm.

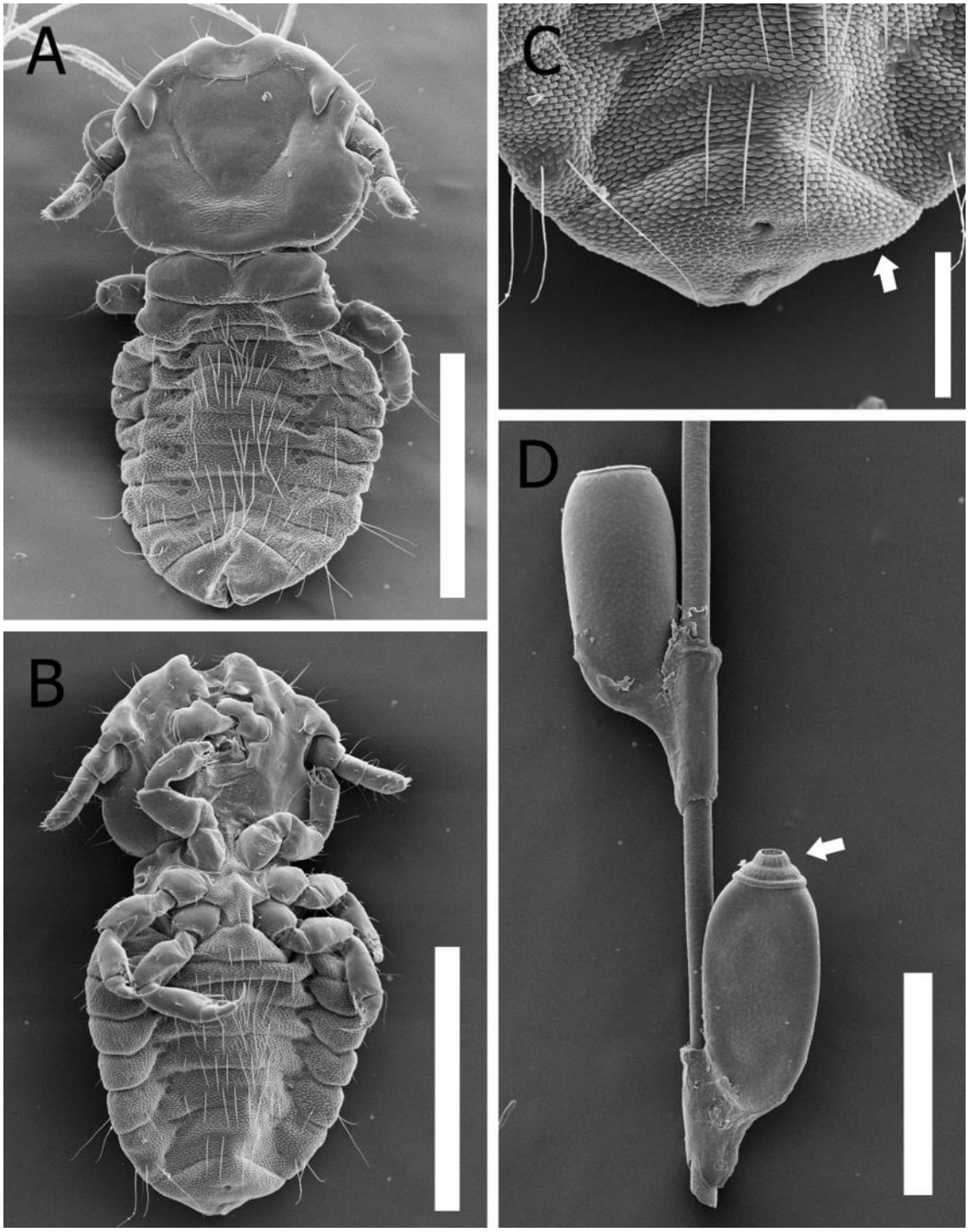

Figure 3. SEM images of the *Neotrichodectes* (*Nasuicola*) *pallidus* (Piaget, 1880) eggs and nymph. **A –** nymph, dorsal; **B –** nymph, ventral; **C -** distal part of the ventral region; **D -** eggs attached to a hair. White arrows highlight the scale-like ornamentation and the protective cover in the anterior region. Scale bars: **A** and **D** 500 μm, **B** 400 μm and **C** 100 μm.

Only one 18S rRNA sequence from one male collected from a coati from PEP was obtained. BLAST results showed 100% query-coverage, 98% identity and 0.0 E-value with *Bovicola bovis* (Linnaeus, 1758) (AY077769), and 90% identity from *F. subrostratus* (AY077770). BI phylogenetic analyses based on the 18S rRNA gene

clustered the obtained sequence in a large clade, composed of *Neotrichodectes arizonae* Werneck 1948, *F. subrostratus*, and *Bovicola ovis* (Schrank, 1781) (**Figure 4A**). Three sequences were obtained from the *cox1* gene from one female, one male and one nymph, collected from animals from PEP. Sequences were 100% identical and presented 99% of query-coverage, 76% identity and 0.0 E-value with *Geomydoecus* sp. (JF342603, JF342605, JF342569). BI phylogenetic analyses based on the *cox-1* gene clustered the sequences of *N. pallidus* in a large clade composed of sequences of different *Geomydoecus* sp., *Thomomydoecus* sp. and *Bovicola caprae* (Gurlt, 1843) (**Figure 4B**). All obtained sequences were deposited at GenBank under accession numbers OP831279 (18SrRNA) and OP852364-OP852366 (*cox1* gene).

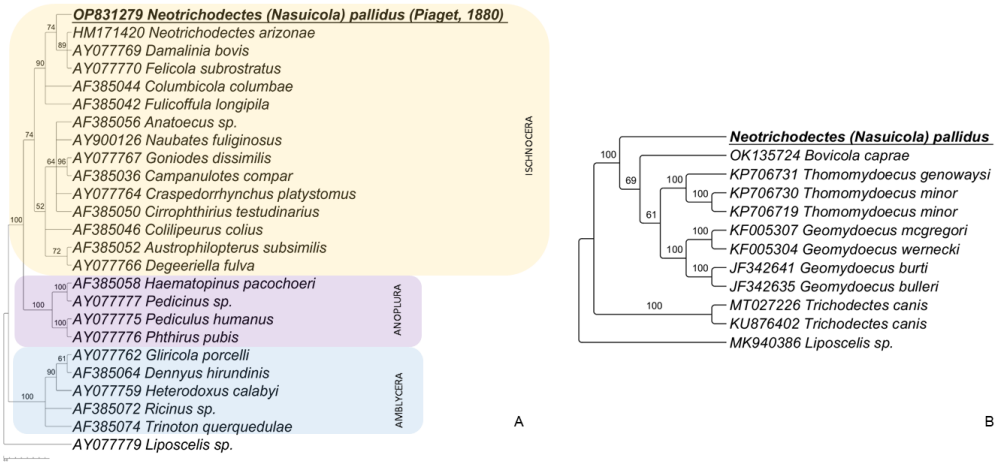

Figure 4. Bayesian phylogenetic trees, with numbers at nodes that corresponding to the bootstrap. Accession numbers are indicated in the sequences. Sequence of *Neotrichodectes* (*Nasuicola*) *pallidus* detected in the present study is highlighted in bold/underlined. *Liposcelis* sp. was used as outgroup. **A)** Alignment of 677 bp fragment 18S rRNA sequences, TN+G4 evolutionary model and gamma shape = 0.22; **B)** Alignment of 495 bp fragment 18S rRNA sequences, TN+G4 evolutionary model and gamma shape = 0.22. *Liposcelis* sp. was used as outgroup.

Discussion

Herein, we expand the Brazilian geographical distribution of *N. pallidus* for the Midwest region of Brazil. This species has been previously recorded in some Brazilian regions with different prevalence. It infested 52.6% (10/19) ring-tailed coatis sampled in a fragment of Atlantic Forest, Juiz de Fora city, Minas Gerais state, Brazil (n=61) (Rodrigues et al., 2006). Picolli (2010) found *N. pallidus* infesting one coati in an area of Atlantic Forest in a border area between the states of Rio Grande do Sul and Santa Catarina, with no mention of the number of specimens. Magalhães-Matos et al. (2022) reported a prevalence of 13% (11/86) in Atlantic Forest (Iguaçu National Park, Foz do Iguaçu city, Paraná state), (n=40; 3 females, 6 males and 31 nymphs). In areas of Atlantic Forest and Cerrado biomes in Mangabeiras Municipal Park, Belo Horizonte city, Minas Gerais state, Estevam et al. (2020) collected 51 specimens (prevalence of infestation was 24.4%). Recently, in *Centro de Endemismo,* an area of Atlantic Forest in the state of Pernambuco, Vale (2020) reported a prevalence of infestation by *N. pallidus* of 54.6% (30/55) among the sampled coatis. These findings suggest that the coatis analyzed in Juiz de Fora (prevalence of 52.6%) and in *Centro de Endemismo* (54.6%)

may have a more important epidemiological role in the maintenance of *N. pallidus* when compared to coatis sampled in other studied regions (prevalence varying from 13-25.75%), including those sampled in present work. Even within the same biome, each location sampled has its own characteristics of climate, phytophysiognomy, faunistic composition and anthropic influence that might influence the differences in the occurrence of parasites.

Clinical signs associated with chewing louse infestation are well known in domestic animals. In dogs and cats, *T. canis* and *F. subrostratus*, respectively, can cause cutaneous crust and hair loss, especially in breeds with long ears (e.g. Basset, Cocker spaniel) and longhair (e.g. Persian cats) (Benelli et al., 2018). In farm animals, *B. bovis* and *B. ovis can cause* cutaneous irritation, itching, and hair loss in several parts of the body of cattle and sheep, respectively (Benelli et al., 2018). Large chewing louse infestations may cause reduced food consumption, and consequently interfere with weight gain *(Kettle, Lukies 1982;* Masutti, Zangheri 2001; *Rony et al., 2010). On the other hand, little is known about the impact of louse infestation on wild animals. In wild birds, previous studies* have reported reductions in the potential attractiveness of louse-infested males to females, since the presence of lice can cause feather damage (Clayton, 1991; Borgia et al., 2004; Garamszegi, et al. 2005). The presence of lice can also affect the metabolic rates of avian hosts. Free-ranging birds can increase metabolic rates and heavily infested birds need to draw on fat reserves to maintain these energetic costs, leading to a chronic decline in body mass over several months (Booth et al., 1993).

To the author's knowledge, there is no information about the impacts and possible clinical alterations due to the presence of *N. pallidus* in procyonids. In this host group, information available is mainly associated with updates on checklists and molecular detection of pathogens in lice (Ford 2004; Reeves et al., 2005; Torres-Mejía, De La Fuente 2006; Ouarti et al., 2021). Estevam et al. (2020) found that 13.2% (20/151) of coatis presented some clinical abnormalities, such as dull and ruffled fur, scars, and other clinical alterations, but with no mention of the presence of lice in those animals. In the present study, although some animals presented skin lesions, they were unlikely to have been associated with louse infestations. The infestation of *N. pallidus* at low levels did not seem to interfere with parasitized coatis´ skin/fur condition. All animals examined presented good body condition, with weight within normal expectations for the species (Barreto et al., 2021, Perles et al., 2022b). It is essential to highlight that none of the captured animals presented high infestation rates. Large infestations can cause problems to the host, such as those already demonstrated in domestic animals (*Kettle, Lukies, 1982;* Masutti, Zangheri 2001; Rony et al., 2010).

We provide the first sequences of two molecular markers and phylogenetic inferences from specimens of *N. pallidus*. Before the advent of molecular techniques, traditional phylogenetics based on morphology suggested that parasitic lice had a monophyletic radiation, mainly because their permanent parasitic lifestyle. Lice were at one time classified into two orders: Mallophaga (all chewing lice) and Anoplura (all sucking lice) (Kim, Ludwig 1978, 1982). Lyal (1985) and Smith (2004) challenged the monophyly of the Mallophaga by supporting a topology of two sister clades, one clade containing the Amblycera, and the other containing the Ischnocera, Rhynchophthirina, and Anoplura. With the advance of sequencing technologies, the evolutionary relationships of lice and their taxonomy have changed considerably over multiple revisions (Boyd, Reed 2012). Johnson and Whiting (2002) and Barker *et al.* (2003) were the first to examine Phthiraptera phylogeny by using molecular data from nuclear and mitochondrial markers and supported the polyphyletic origin. Despite

studies regarding phylogenetic analyses of lice parasitizing domestic animals, there is a paucity of information on molecular data from lice from wild animals, especially from Brazil. Sequences from only one louse species *Neotrichodectes arizonae* Werneck, 1948 (18S rRNA, *cox1* and EF1-alpha genes), a parasite of the American hog-nosed skunk (*Conepatus leuconotus* (Lichtenstein, 1832), are available in the GenBank database. All previous studies on lice from coatis in Brazil reported only prevalence and life stages, with no attempts to perform molecular inferences of the louse species. Future molecular studies, aiming at achieving sequences of both nuclear and mitochondrial genes from lice found parasitizing wild animals should be performed, to achieve an increased database to allow new comparative and evolutionary relationships within Phthiraptera.

Conclusions

The present work extends the Brazilian geographic range of distribution for *N. pallidus*, and includes the first molecular contribution with sequencing and phylogenetic inference based on two molecular markers (18S rRNA and *cox1*). Also, for the first time, SEM images from all life stages (eggs, nymphs and adults from both sexes), with the first morphological description of eggs. The infestation by *N. pallidus* with low parasite burden did not seem to produce any alterations on body condition and overall skin/fur condition of the sampled coatis in Midwestern Brazil.

Ethics Statement

All methods were carried out in accordance with relevant guidelines and regulations and were approved by the "Instituto Chico Mendes de Biodiversidade" (ICMBio, protocol number 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP, protocol number 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB, protocol number 001/2018) and Air force cooperation agreement (N°01/GAP-CG/2018).

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Capítulo 4* - Longitudinal dynamics and health impact of *Hepatozoon procyonis* (Apicomplexa: Hepatozoidae) on naturally infected ring-tailed coatis *Nasua nasua* (Carnivora: Procyonidae) from Midwestern Brazil

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Abstract

This study aimed to morphologically and molecularly detect *Hepatozoon procyonis* in ring-tailed coatis' (*Nasua nasua*) blood and associated ticks from central-western Brazil, Campo Grande, Mato Grosso do Sul state and also evaluate the impact of the protozoa in blood parameters and coati´s health. Samplings were performed in a conservation area *Parque Estadual do Prosa* (PEP) and in a Brazilian Air Force Private Area namely *Vila da Base Aérea* (VBA), between March 2018 and April 2019. We collected 165 blood samples, 61 from recaptured coatis. Peripheral blood smears were stained with Romanovsky-type stain for *H. procyonis* parasitemia assessment. DNA extracted from blood samples and ticks (*Amblyomma* spp.) were submitted to a nested PCR (nPCR) assay based on the 18S rRNA gene for *Hepatozoon* spp. Out of 104 individuals sampled, 80 (77%) were positive for *H. procyonis* in at least one capture. Overall, 67/165 (40.6%) blood smears showed *H. procyonis* gametocytes (PEP: 41/63 - 65%; VBA: 26/102 - 25.5%). Parasitemia based on 500 assessed leucocytes ranged from 1 (0.2%) to 50 (10%) and 1 (0.2%) to 25 (5%), from animals sampled in PEP and VBA, respectively. Fluctuation on the parasitemia was observed during recaptures. nPCR results showed higher positivity when compared to blood smears, i.e. 112/165 (68%) positive blood samples [PEP: 41/63 (65%), VBA: 26/102 (25.5%)]. In total, 63/248 (25.4%) tick DNA samples were positive at nPCR for *Hepatozoon* sp., including 32/87 (37%) pools (1 to 10 larvae) of *Amblyomma* larvae, 21/105 (20%) pools (1 to 5 nymphs) of *Amblyomma sculptum* nymphs, 9/43 (21%) pools (1 to 5 nymphs) of *Amblyomma dubitatum*nymphs, and 1/12 (8%) *A. sculptum* adult female. The partial 18S rRNA sequence from one coati's blood sample and one representative of each positive tick species randomly selected from each area for sequencing (1,000 bp) showed 100% identity with sequences of *H. procyonis* from GenBank previously detected in coatis. Regarding *H. procyonis* infection, no statistical differences were obtained when comparing males vs. females (*p-value* 0.67), immature animals vs. adults (*p-value* 0.31), rainy vs. dry season (*p-value* 0.51) and sampling location (*p-value* 0.42). No noticeable alteration in blood parameters or heath status was observed in parasite animals. *H. procyonis* circulates in a high prevalence in coatis from central-western Brazil. Parasitemia fluctuates among different coatis' recaptures and apparently the infection has no influence in coatis' hematological and clinical parameters.

Key-words: Hepatozoonosis; 18S rRNA; Parasitemia; Procyonids; Wildlife conservation

1. Introduction

Protozoa of the genus *Hepatozoon* comprise hemogregarinids belonging to the phylum Apicomplexa with a heteroxene life cycle (Smith, 1996). The intermediate hosts are represented by vertebrates that usually acquires the infection by ingesting blood-sucking arthropods (ticks, flies, mosquitoes), which in turn play a role as sporulated oocysts-containing definitive hosts (Smith, 1996).

Even though *Hepatozoon* spp. have been described parasitizing a wide variety of vertebrate hosts all over the world, few are the reports of *Hepatozoon* in animals from the family Procyonidae. Richard (1961) described, for the first time, the infection of *Hepatozoon procyonis* in animals from this group of mammals. The gametocytes found in monocytes of raccoons (*Procyon lotor*) in southwestern Georgia, USA, showed sausage-shaped structures, encased in a capsule with a narrow, recurved extension at one end – "recurved tail", which represents the main morphological characteristic of *H. procyonis* gametocytes (Richard, 1961). Since its first description, *H. procyonis* has been reported in other animals from the family Procyonidae, such as raccoons from other regions of the USA (Clark et al., 1973; Schaffer et al., 1978; Hanion et al., 1989) and Costa Rica (Meneses et al., 2016), as well as in crab-eating raccoons (*Procyon cancrivorous*) from Panama (Schneider, 1968).

In Brazil, *H. procyonis* gametocytes were firstly detected in blood smears from 2/20 ring-tailed coatis (*Nasua nasua*) and 1/1 crab-eating raccoons in the state of Rio de Janeiro (Rodrigues et al., 2006). Interestingly, even though the authors found gametocytes in *P. cancrivorous*' monocytes, the parasites were most often found in neutrophils. This finding was strikingly different from those observed by Richard (1961) in *P. lotor*. Eleven years later, Sousa et al. (2017) characterized, for the first time, *H. procyonis* 18S rDNA fragments detected in ring-tailed coatis sampled in southern Pantanal, Mato Grosso do Sul state, Midwestern Brazil. Phylogenetic inferences clustered the sequences obtained in coatis in a large clade with sequences of *Hepatozoon felis, Hepatozoon canis* and *Hepatozoon americanum* (Sousa et al., 2017). However, no gametocytes were found in blood smears of the sampled ring-tailed coatis. After those reports, da Silva et al. (2018) performed the redescription of *H. procyonis* in coatis from São Paulo State, southeastern Brazil, using both morphological and molecular tools. Recently, Estevam et al. (2020) monitored a population of coatis during seven years in the state of Minas Gerais. The above-mentioned authors performed both molecular and

parasitological tests (blood smears) as well as clinical evaluation, such as body condition, mucous membrane coloration and fur condition. Even though the authors observed that 13% (20/151) ring-tailed coatis presented some clinical alterations, such as dull and ruffled fur, pale mucosa, scars and swollen lymph nodes, no association between infection by *H. procyonis* and health status was reported.

Even though infections by *Hepatozoon* spp. in wild animals seem to be associated with subclinical course of the disease (Kocan et al., 2000; Metzger et al., 2008), some studies have shown the occurrence of clinical disease in coyotes (*Canis latrans*) in the USA (Kocan et al., 2000) and mortality of hyenas (*Crocuta crocuta*) in Tanzania (East et al., 2008). Alic et al. (2022) described a fatal case caused by coinfection by *Leptospira* spp. and *H. canis* in a red fox cub (*Vulpes vulpes*) in Saravejo, Bosnia and Herzergovina. These protozoans may play a role as a potential opportunistic pathogen in immunocompromised animals, in stressful situations or in coinfections (Baneth and Weigler, 1997, Kubo et al., 2006).

Although it has been shown that *H. procyonis* occurs in different states from Brazil (Rodrigues et al., 2006; da Silva et al., 2018, Sousa et al., 2017; Estevam et al., 2020), little is known about its infection dynamics in naturally infected animals and the consequences for the host. The present study aimed to: i.) monitor the fluctuation of *H. procyonis* parasitemia in free-living ring-tailed coatis captured every three months during an 11-month longitudinal study in two spots in central-western Brazil; ii.) investigate the fluctuation of *H. procyonis* DNA in blood samples and tick specimens obtained from the sampled coatis in the period of study; iii.) assess the impact of *H. procyonis* infection on coatis' hematological and health parameters.

2. Materials and Methods

All experimental procedures were approved by the "Instituto Chico Mendes de Biodiversidade" (ICMBio, protocol number 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP, protocol number 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB, protocol number 001/2018) and Air force cooperation agreement (N°01/GAP-CG/2018).

2.1. Study sites and sampling

Free-living ring-tailed coatis were captured in two areas located in Campo Grande city, Mato Grosso do Sul State, midwestern Brazil (Fig. 1). The first sampling

spot was *Parque Estadual do Prosa* (PEP) (20.44987 S, 54.56529 W), which has an approximate area of 135 acres and it is located on the plateau of Serra de Maracajú. This area has a Wild Animal Center that usually receives free-living animals belonging to various species. Two density studies were performed at PEP with coatis, where 33.7 individuals/km^2 were estimated, equivalent to 120 animals in the study area (Costa and Mauro, 2008) and 11.2 individuals/ km^2 (30 animals in the study area) (Barreto et al., 2021). The second sampling spot was Brazilian Air Force Private Area (VBA) (20.47163 S, -54.65405 W), a Brazilian Air Force base. This area has military buildings, and owned cats and dogs. Only one density study was performed at VBA, where 19.4 individuals / km^2 were estimated, equivalent to 41 animals in the study area (Barreto et al., 2021).

Fig. 1. Aerial view of *Parque Estadual do Prosa* (PEP) and *Vila da Base Aérea (*VBA) (blue arrows), Campo Grande (red arrow), Mato Grosso do Sul state, midwestern Brazil (Source: Google Earth).

The coatis were captured every three months during 10 consecutive nights (with interval of two days - Saturday and Sunday) between March of 2018 and January of 2019, totalizing 4 sampling campaigns in PEP and 5 in VBA. All captures and recaptures were performed by convenience, and recaptures were performed by chance. Animals were captured using metal traps (1 m x 0.40 m x 0.50 m) placed arbitrarily according to the possibility of human access and availability of shadow, in order to cover most of the PEP and VBA areas. They were anesthetized with an association of Tiletamine hydrochloride and Zolazepam hydrochloride (Telazol, Zoetis® - 7 mg/Kg, Intramuscularly). After chemical restraint, animals were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue between the shoulder blades (Perles et al., 2022). They were measured and the age was estimated according to Olifiers et al. (2010). Blood was sampled from femoral

vein, placed in tubes containing EDTA (Ethylenediamine tetraacetic acid), and divided into two aliquots: one used for hematological analyses and the other was placed in RNAse/DNAse free cryotubes and stored in an -80°C freezer until molecular analyses. In total, 165 blood samples were obtained (63 in PEP and 102 in VBA). In PEP, samples were obtained from 47 different individuals (27 females and 20 males; 7 infants, 2 subadults and 38 adults). One sampling procedure was performed in 33/47 individuals (one sampling), two sampling procedures were performed in 12/47 coatis (first sampling and one recapture), and 2/47 animals were recaptured twice (first sampling and two recaptures), totalizing 63 coatis' blood samples in PEP. In VBA, samples were obtained from 57 different individuals (32 females and 25 males; 11 infants, 8 subadults and 38 adults). One sampling procedure was performed in 34/57 individuals (one sampling), two sampling procedures were performed in 12/57 coatis (first sampling and one recapture), 5/57 were recaptured twice (first sampling and two recaptures), 3/57 were recaptured three times (first sampling and three recaptures), 1/57 were recaptured four times, and 2/57 were recaptured five times, totalizing 102 samples from VBA.

Additionally, the sampled coatis had their entire body inspected for the presence of ectoparasites. Ticks were stored in 100% ethanol (Merck Ensure®) in RNAse/DNAse-free microtubes. Taxonomic identification of ectoparasites was performed with a stereoscopic microscope (Olympus SZX7) following previously published identification keys (Martins et al., 2010, 2016; Dantas-Torres et al., 2019). After taxonomic identification, the microtubes were stored in an -80°C freezer until molecular analyses. In total, 2,241 ticks (715 larvae, 1,298 nymphs and 12 adults) were collected at the present study in both areas, including animals from the first capture and recaptures. Larvae were identified to genus level and all the nymphs and adults were identified to species level (Perles et al., 2022).

2.2. Blood smears

Peripheral blood smears (ear tip) were performed for each sampled coati. The blood smears were stained by a quick Romanovsky-type stain (Laborclin®, São Paulo, Brazil) in order to detect the presence of *H. procyonis* gametocytes. The slides were evaluated at 1,000 x magnification (immersion oil) under a light microscope (Olympus BX43) and parasitemia was counted in 500 leukocytes (Aktas et al., 2015, da Silva et al., 2018).

2.3. Clinical parameters

In order to check the health condition of each captured animal, lymph nodes palpation (popliteal and submandibular), oral mucous coloration, skin turgor and overall fur/skin condition were accessed. We also recorded clinical abnormalities.

2.4. Hematological analyses

Red blood cells (10^6/µL), hemoglobin (g/dL), hematocrit (%), mean corpuscular hemoglobin concentration (MCHC - g/dL), mean corpuscular value (MCV - fL), platelets (mm^3) and leukocytes (mm^3) were determinate using hematology analyzer device (POCH-iV 100, Syxmex®, Brazil), standardized for ring-tailed coatis according to manufactures instructions.

2.5. *DNA extraction from coatis' blood samples and conventional PCR (cPCR) for endogenous gene (*gadph*)*

DNA was extracted from 200 µL of blood using Illustra Blood Mini Kit (GE Healthcare®, USA), according to the manufacturer's instructions. The DNA concentration and absorbance ratio (260/280 nm) were measured using a spectrophotometer (Nanodrop, Thermo Scientific, USA). To evaluate the quality of the extracted DNA and avoid false negative results, DNA samples were tested by a conventional PCR targeting the mammal *gapdh* (Glyceraldehyde 3-phosphate dehydrogenase), using the primers GAPDH-F (5´ CCTTCATTGACCTCAACTACAT 3´) and GAPDH-R (5´ CCAAAGTTGTCATGGATGACC 3´) (Birkenheuer et al., 2003). Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in PCR assays.

2.6. DNA extraction from ectoparasites and PCR for endogenous gene (16S rRNA)

DNA was extracted from ticks' larvae, nymphs and adults. DNA from larvae was extracted in pools up to 10 individuals collected from the same host; DNA from nymphs was extracted in pools up to 5 individuals collected from the same host; DNA was extracted from tick adults individually. Initially, to remove the alcohol from the ectoparasites, all larvae and nymph pools and individual adults were washed in a solution of PBS 1x pH 7.2 and then DNA was extracted using Biopur Mini Spin Plus kit (Mobius®, Brazil), according to the manufacturer's instructions. The DNA concentration

and absorbance ratio (260/280 nm) were measured using a spectrophotometer (Nanodrop, Thermo Scientific, USA). To evaluate the quality of the extracted DNA and avoid false negative results, DNA samples extracted from ticks were tested by a conventional PCR targeting the 16S rRNA gene, using primers 16S-F (5´CCGGTCTGAACTCAGATCAAGT 3´) and 16SR (5´ CTGCTCAATGATTTTTTAAATTGCTGTGG 3´) (Black and Piesman, 1994). Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in PCR assays. One positive sample from 16S rRNA gene from each obtained nymphae species were sequenced in order to confirm morphological identification.

2.7. *Nested-PCR (nPCR) for* Hepatozoon *spp. based on the 18S rRNA gene*

Gapdh and 16S rRNA positive samples from coatis' blood and ectoparasites DNA, respectively, were submitted to a nested PCR (nPCR) that amplifies a fragment of 1,120 pb of the 18S rRNA gene, using primers HAM-1 (5′ GCCAGTAGT CATATGCTTGTC 3′) and HPF-2 (5′ GACTTCTCCTTCGTCTAAG 3′) (Criado-Fornelio et al., 2006) on the first round and 4558 (5′ GCTAATACATGAGCAAAA TCTCAA 3′) and 2733 (5′ CGGAATTAACCAGACAAAT 3′) (Mathew et al. 2000) on the second round. Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in PCR assays. *Hepatozoon* sp. DNA detected in a naturally infected maned-wolf (*Chrysocyon brachyurus*) was used as positive control (Perles et al., 2019).

2.8. Agarose gel electrophoresis and sequencing

The results of PCR and nPCR assays were visualized in 1% agarose gel stained by ethidium bromide solution and results were visualized in UV transilluminator (ChemiDoc MP Imaging System, Bio Rad®). One positive sample from each spot (PEP and VBA) and one positive sample from each tick species were sequenced. The sequencing of amplicons was realized by Sanger method (Sanger et al., 1977) using ABI PRISM 3730 DNA Analyzer (Applied Biosystems) at Human Genome and Stem Cell Research Center, "Instituto de Biociências", University of São Paulo (USP), Brazil.

2.9. Statistical analyses

All analyzes were performed using the R Software, using a significance level equal to 5%. The effect of the covariates namely i) season (rainy vs. dry season); ii) location (PEP vs. VBA); iii) gender (female vs. male); and iv) age group (immature x mature) on the positivity for *H. procyonis* by PCR was assessed using a binomial logistic regression. Infants and subadults were joined in a category since we obtained few animals from these groups. This analysis was applied on data obtained from animals captured only once (first capture – animals with recaptures were not included since they those procedures were performed by convenience with different time intervals among them; they were included in a subsequent analyses). To apply this model, the absence of multicollinearity between the independent variables was verified. The odds ratio values and their respective confidence intervals were obtained based on the exponential value of the estimates of the model.

Descriptive analysis of the blood variables evaluated in the study was performed considering the mean and standard deviation values. The effect of PCR and sex factors in the animals captured only once was obtained by an analysis of variance with two independent factors (Two-way ANOVA). The contrasts within the factors were obtained using the Bonferroni multiple comparisons test. Gender factor was tested separately, since Macedo et al. (2022 – submitted; Personal communication: Prof. Heitor Miraglia Herrera) showed statistical differences between hematological analyses between males and females. To verify the effect of the capture factor (first, second, third, fourth and fifth capture) on blood variables, data were analyzed using the Linear Mixed Model (LMM), considering animals as a random effect. The standardized residues in both models were considered normal by the Shapiro-Wilk test and homoscedasticity met by the Levene´s test. Data with standardized residues greater than 3 times the interquartile range were considered outliers and removed from the analyses.

To analyze all recaptured animals, Principal Component Analyses (PCA), which was based on a correlation matrix between blood variables of data and capture and recapture, was performed. Confidence ellipses indicate the degree of clustering of the evaluated groups based on a coefficient of 0.95.

3. Results

3.1. Blood smears

Out of 165 blood smears analyzed in both areas, 67 (40.6%) presented *H. procyonis* gametocytes (Table 1 - Supplementary file). Parasitemia counted in 500 leucocytes ranged from 1 (0.2%) to 50 (10%). Sixty-six samples presented gametocytes in monocytes and neutrophiles, and one animal presented extracellular gametocytes.

3.1.1. Parque Estadual do Prosa (PEP)

In total, 41/63 (65%) of blood smears from PEP had gametocytes, with parasitemia ranging from 1 (0.2%) to 50 (10%) in 500 leucocytes (Table 1 - Supplementary file). Positive blood smears were obtained from 32/47 (68%) different coatis (19 females and 13 males – two infants, two subadults, and 28 adults). Twenty smears (20/41 – 49%) were obtained from coatis sampled only one time, and thus, it was not possible to follow parasitemia during time (recaptures). Twenty-one smears (21/41 – 51%) were obtained from recaptures of 12 different coatis, in which it was possible to follow parasitemia during recaptures (Table 1 – Supplementary file).

From recaptured animals, 3/12 (PEP 01, 17 and 24) did not presented gametocytes on the first blood smear, becoming positive on the second (PEP 17 and 24) and third (PEP 01) sampling. Only one animal (1/12) (PEP 05) was positive on the first blood sampling, and negative on the second sampling. The eight remaining animals (8/12) were positive on the first and all subsequent samplings, with parasitemia ranging from 1 (0.2%) to 39 (8%) (Table 1 – Supplementary file).

Table 1. Parasitemia and nPCR positivity for *Hepatozoon procyonis* from ring-tailed coatis (*Nasua nasua*) sampled at *Parque Estadual do Prosa* (PEP), in Mato Grosso do Sul state, Brazil, throughout recaptures, between March of 2018 and January of 2019.

		1° Sampling		**2° Sampling**		**3° Sampling**	
	Gender	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**
PEP 01	M[1]	0	Positive	0	Positive	8/500 (1.6%)	Positive
PEP 02	M	2/500 (0.4%)	Positive	8/500 (1.6%)	Positive	-	-
PEP 03	F[2]	2/500 (0.4%)	Positive	31/500 (6.2%)	Positive	-	-
PEP 04	M	23/500 (4.6%)	Positive	1/500 (0.2%)	Positive	5/500 (1%)	Positive
PEP 05	M	3/500 (0.6%)	Positive	0	Negative	-	-
PEP 12	F	11/500 (2.2%)	Positive	16/500 (3.2%)	Positive	-	-

		1° Sampling		2° Sampling		3° Sampling	
	Gender	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**
PEP 17	F	0	Negative	7/500 (1.4%)	Positive	-	-
PEP 20	F	6/500 (1.2%)	Positive	39/500 (7.8%)	Positive	-	-
PEP 23	F	1/500 (0.2%)	Positive	1/500 (0.2%)	Positive	-	-
PEP 24	F	0	Positive	9/500 (1.8%)	Positive	-	-
PEP 31	M	3/500 (0.6%)	Positive	18/500 (3.6%)	Positive	-	-
PEP 43	M	2/500 (0.4%)	Positive	2/500 (0.4%)	Positive	-	-

3.1.2. Vila da Base Aérea (VBA)

In total, 26/102 (25.5%) of blood smears from VBA showed *H. procyonis* gametocytes, with parasitemia ranging from 1 (0.2%) to 25 (5%) in 500 leucocytes (Supplementary file 1). Positive blood smears were obtained from 18/57 (31%) different coatis (8 females and 10 males – 5 infants, 3 subadults, and 10 adults). Nine (9/26 – 35%) positive blood smears were obtained from coatis sampled only one time, and thus, precluding following parasitemia during time (recaptures). Seventeen blood smears (17/26 – 65%) were obtained from nine different coatis that were recaptured, following parasitemia during recaptures (Table 2). Regarding recaptured animals, 6/18 (VBA 01, 03, 17, 19, 25 and 39) did not present *H. procyonis* gametocytes on the first blood smears, being positive on the second (VBA 01, 17, 19 and 39), third (VBA 25), and sixth sampling (VBA 03). Two animals (VBA 21 and 38) were positive on the first sampling and in all successive samplings, except animal VBA 21, which did not present gametocytes on the third sampling. Also, this animal presented extracellular gametocytes on the second sampling. One animal (VBA 07) showed gametocytes on the first and fourth samplings, and was negative on the second and third (Table 2).

Table 2. Parasitemia and nPCR positivity for *Hepatozoon procyonis* from ring-tailed coatis (*Nasua nasua*) sampled at *Vila da Base Aérea* (VBA), in Mato Grosso do Sul state, Brazil, throughout recaptures, between March of 2018 and January of 2019.

		1° Sampling		2° Sampling		3° Sampling		4° Sampling		5° Sampling		6° Sampling	
	Gender	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**	**Blood smear**	**Nested-PCR**
AFPA	M[1]	**0**	Negative	2/500 (0.4%)	Positive	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
AFPA	F[2]	0	Negative	0	Positive	0	Positive	0	Positive	0	Positive	1/500	Positive
AFPA	M	0	Positive	0	Positive	-	-	-	-	-	-	-	-
AFPA	M	0	Positive	0	Positive	-	-	-	-	-	-	-	-
AFPA	M	3/500	Positive	0	Positive	0	Negative	3/500 (0.6%)	Positive	-	-	-	-
AFPA	F	0	Negative	0	Negative	0	Positive	-	-	-	-	-	-
AFPA	M	0	Positive	0	Negative	0	Positive	0	Positive	-	-	-	-
AFPA	F	0	Negative	0	Negative	0	Positive	-	-	-	-	-	-
AFPA	M	0	Negative	0	Positive	0	Negative	-	-	-	-	-	-
AFPA	F	0	Negative	0	Positive	-	-	-	-	-	-	-	-
AFPA	F	0	Negative	0	Positive	0	Negative	0	Positive	-	-	-	-
AFPA	F	0	Negative	3/500 (0.6%)	Positive	-	-	-	-	-	-	-	-
AFPA	F	0	Negative	1/500 (0.2%)	Positive	-	-	-	-	-	-	-	-
AFPA	M	2/500	Positive	3/500 (0.6%)	Positive	0	Positive	24/500 (4.8%)	Positive	11/500	Positive	2/200	Positive
AFPA	F	0	Negative	0	Positive	-	-	-	-	-	-	-	-
AFPA	F	0	Negative	0	Positive	-	-	-	-	-	-	-	-
AFPA	M	0	Negative	0	Positive	25/500 (5%)	Positive	1/500 (0.2%)	Positive	23/500	Positive	-	-
AFPA	M	0	Positive	0	Negative	0	Positive	-	-	-	-	-	-
AFPA	F	5/500 (1%)	Positive	1/500 (0.2%)	Positive	-	-	-	-	-	-	-	-
AFPA	F	0	Positive	1/500 (0.2%)	Positive	-	-	-	-	-	-	-	-
AFPA	F	0	Positive	0	Negative	-	-	-	-	-	-	-	-
AFPA	M	0	Positive	0	Negative	-	-	-	-	-	-	-	-

3.2. Nested-PCR (nPCR) results for blood samples

All DNA extracted from coatis' blood samples were positive for *gapdh* gene and were submitted to a nPCR for 18S rRNA of *Hepatozoon* sp. The average concentration of the extracted DNA from blood samples was 40 ng/µL (ranging from 7.6 to 106.4 ng/µL), 260/230 mean ratio was 1.7 (ranging from 0.7 to 3.0) and 260/280 mean ratio was 1.7 (ranging from 0.5 to 3.4). Out of 165 samples (63 PEP and 102 VBA), 112 (68%) were positive at nPCR.

3.2.1. Parque Estadual do Prosa

Positive samples at nPCR were obtained from 34/47 (72%) different coatis (20 females and 14 males). Were sampled only once 22/34, and thus, precluding following nPCR results during time (recaptures). Twelve animals were recaptured, allowing following nPCR results during recaptures. Out of 63 blood samples from PEP, 46 (70%) were positive at nPCR. From 46 positive samples at nPCR, 41 (89%) also presented gametocytes of *H. procyonis* on blood smears. Five samples (5/46 - 11%) were positive at nPCR and negative at blood smears. None animal presented gametocytes and was negative at nPCR (Supplementary file 1; Table 1).

Regarding 12 recaptured animals, 11 (92%) were positive at nPCR and 9 (8%) showed gametocytes at blood smear at the first sampling. Animal PEP 17 was negative both in nPCR and blood smear on the first sampling. Two animals (PEP 01 and PEP 24) did not present gametocytes on blood smears, but were positive at nPCR (first sampling) (Table 1). On the second sampling (first recapture), 11/12 were positive at nPCR and only one was negative (PEP 05). Two animals did not show gametocytes (PEP 01 and PEP 05), including PEP 05, which was negative at nPCR. On the third sampling (second recapture), both animals were positive at nPCR and had gametocytes on blood smears (Table 1).

3.2.2. Vila da Base Aérea

Positive samples at nPCR were obtained from 46/57 (81%) different coatis (27 females and 19 males). In total, 24/46 were sampled only once, and thus, precluding following nPCR results during time (recaptures). Twenty-two animals were recaptured, allowing following nPCR results during recaptures (Table 2). Out of 102 coatis' blood samples from VBA, 66 (65%) were positive at nPCR. Out of 66 positive samples at nPCR, 26 (39%) also presented gametocytes of *H. procyonis* on blood smears. Forty samples (61%)

were positive at nPCR and did not showed gametocytes on blood smears. None animal presented gametocytes and was negative at nPCR (Supplementary information 1).

Regarding 22 recaptured animals, 10 were positive at nPCR and 3 showed gametocytes on blood smears on the first sampling (VBA 7, VBA 21 and VBA 38). On the first recapture (second sampling), 6/22 were negative at nPCR and among the 16 positives, only 6 showed gametocytes on blood smears. Animal VBA 03 was negative on blood smears and at nPCR between the first and fifth sampling, and turned out positive at nPCR and blood smears on the sixty-sampling procedure. Animal VBA 21 was positive at nPCR in all six samplings, but did not presented gametocytes on the third sampling. Animal VBA 25 was negative at nPCR/blood smear on the first sampling, then turned out positive at nPCR on the second sampling, and then remained positive (nPCR and blood smears) on the three subsequent samplings (Table 2).

3.3. Statistical analyses for gender, age, season and location (considering non-recaptured animals) for nPCR results

No factor showed significant difference ($p<0.05$) in the binomial logistic regression model (Table 2 - Supplementary file). These findings mean that the difference in the proportion of *H. procyonis*-positive coatis within each factor (i.e. M and F) was a result of chance. The confidence interval was very wide (CIsup – ICinf) for all factors. One suggestion to reduce this CI and, consequently, visualize an association between nPCR-positive animals and the factors is to increase the sample size, which sometimes is not simple to achieve when working with free-living animals.

3.4. Nested-PCR (nPCR) results for ectoparasites

It was obtained a total of 265 extracted DNA samples from ectoparasites (pools and individual adults – 87 pools of *Amblyomma* sp. larvae; 105 pools of *Amblyomma sculptum* Berlese 1888 nymphs; 43 pools of *Amblyomma dubitatum* Neumann 1899 nymphs and 12 adults – 2 females *Amblyomma ovale* Koch 1884, 2 male *A. ovale,* 5 *A. sculptum* female*,* 3 *A. sculptum* male). The average concentration of the extracted DNA was 24 ng/µL (ranging from 0.8 to 202 ng/µL), 260/230 mean ratio was 2.4 (ranging from 1.0 to 7.3) and 260/280 mean ratio was 1.5 (ranging from 0.04 to 2.8). At the PCR targeting the endogenous gene, 248 (94%) were positive for 16S rRNA gene and then submitted to nPCR for *Hepatozoon* sp. (nine *Amblyomma* sp. larvae pools, five *A. sculptum* nymphs and three *A.*

dubitatum nymphs were negative, and then excluded from the nPCR). In total, 63/248 (25.4%) extracted DNA were positive at nPCR, including: a) 32/87 (37%) pools of *Amblyomma* sp. larvae; b) 21/105 (20%) pools of *A. sculptum* nymphs; c) 9/43 (21%) pools of *A. dubitatum* nymphs; d) 1/12 (8.3%) adult was positive (*A. sculptum* adult female).

3.5. Sequencing results

Regarding ectoparasites identification, the sequences obtained from *A. sculptum* and *A. dubitatum* specimens were deposited in GenBank under accession numbers MT974144 and MT974145. One positive sample for *Hepatozoon* sp. nPCR obtained from a coati's blood aliquot in each sampling spot (PEP and VBA) was randomly selected for sequencing. Also, one positive sample obtained from pools of *Amblyomma* sp. larvae, pools of *A. sculptum* nymphs, and pools of *A. dubitatum* nymphs (randomly selected since no sampling spot was considered) was sequenced. The obtained sequences were submitted at GenBank (accession numbers: MT102405; MW862003; MW862004; MW862005; MW86006) and showed 100% of query coverage and identity and 0.0 of E.value with sequences of *H. procyonis* from GenBank (accession numbers: MF685386-MF685410 and KX776359-KX776377).

3.6. Clinical parameters and Hematological analyses

3.6.1. Non-recaptured animals

Out of the 33 individuals that were not recaptured in PEP, 20/33 (60.6%) were positive for *H. procyonis* by blood smears analysis [parasitemia ranging from 1 (0.2%) to 50 (10%)] and 22/33 (66.6%) were positive by nPCR. Among the 33 coatis, only two (6%) presented alteration in the oral mucous coloration, with one presenting cyanotic membrane (PEP 13 – parasitemia 1.8% and positive at nPCR) and one presenting pale mucous (PEP 25- parasitemia 3.6% and positive at nPCR). All 31 remaining coatis had normal coloration of oral mucous. Out of the 34 individuals that were not recaptured in VBA, 9 (26.5%) were positive by blood smears analysis [parasitemia ranging from 1 (0.2%) to 11 (2 %)] and 23 (68%) were positive by nPCR. Among the 33 coatis (one positive animal was not recorded – VBA 59), four had pale oral mucous; all these four coatis did not present *H.*

procyonis gamonts on blood smears analysis and three were positive for *Hepatozoon* spp. by nPCR. All 29 remaining animals had normal coloration of oral mucous.

From non-recaptured animals from PEP, only two had swollen popliteal lymph nodes (PEP 47 and PEP 53). Both were positive for *Hepatozoon* sp. by blood smears and nPCR analyses. All remaining animals (positive and negative for *Hepatozoon*) had normal lymph nodes at palpation. From non-recaptured animals from VBA, only two had swollen popliteal lymph nodes (VBA 34 and VBA 43). Both were negative for *Hepatozoon* on blood smears analysis and one was positive at nPCR. All remaining animals (positive and negative for *Hepatozoon*) had normal lymph nodes at palpation.

3.6.2. *Statistical analyses comparing positive x negative animals for* Hepatozoon *regarding blood parameters among non-recaptured animals*

There was no statistical difference between blood parameters from positive vs. negative animals captured only once (Table 3). Since it has been demonstrated statistical difference in blood parameters from ring-tailed coatis between female and males (unpublished; personal communication Dr. Heitor Miraglia Herrera), we decided to analyze gender separately in the present study. Considering that no statistical difference was observed comparing both studied areas (PEP x VBA), we decided to analyze blood parameters from both areas together. Regarding the erythrocyte variable, the nPCR factor (positive x negative) had a *p-value* of 0.422 and the gender factor had a *p-value* of 0.0015. In the hemoglobin variable, the nPCR factor had a *p-value* of 0.274 and the gender factor had a p-value of 0.0011. In the hematocrit variable, the PCR factor had a *p-value* of 0.253 and the gender factor had a *p-value* of 0.0002. In the variable VCM, the nPCR factor had a p-*value* of 0.423 and the gender factor had a *p-value* of 0.463. In the CHCM variable, the nPCR factor had a *p-value* of 0.857 and the gender factor had a *p-value* of 0.558. In the platelet variable, the nPCR factor had a *p-value* of 0.700 and the gender factor had a *p-value* of 0.417. In the variable leukocytes, the nPCR factor had a *p-value* of 0.693 and the gender factor had a *p-value* of 0.713 (Table 3). Therefore, no statistical difference was found between nPCR positive x negative animals, but there are statistical differences among females x males' erythrocyte counts, hemoglobin concentration and hematocrit value.

Table 3. Descriptive analyses of hematological parameters comparing positive vs. negative coatis for 18S rRNA of *Hepatozoon procyonis*.

Variable	PCR	Sex	n	Mean	SD	Min	Max
Red Blood cells (RBC (10^6/µL)	Negative	F	10	5,24 ^{A,a}	0,22	4,79	5,62
	Positivo	F	29	5,29 ^{A,a}	0,40	4,42	6,04
	Negative	M	9	5,51 ^{A,b}	0,40	5,08	6,15
	Positivo	M	16	5,66 ^{A,b}	0,46	4,90	6,44
Hemoglobin (g/dL)	Negative	F	11	10,05 ^{A,b}	0,60	9,00	11,20
	Positivo	F	30	10,23 ^{A,b}	0,91	8,40	12,00
	Negative	M	9	10,70 ^{A,b}	0,73	9,50	11,60
	Positivo	M	16	11,11 ^{A,b}	1,21	9,50	13,30
Hematocrit (%)	Negative	F	9	30,81 ^{A,b}	1,28	28,40	33,20
	Positivo	F	30	31,42 ^{A,b}	2,29	26,10	36,40
	Negative	M	9	32,89 ^{A,b}	1,35	30,80	34,70
	Positivo	M	16	33,77 ^{A,b}	2,96	29,10	38,20
MCV (fL)	Negative	F	10	58,40 ^{A,a}	2,46	54,30	63,20
	Positivo	F	29	59,97 ^{A,a}	2,72	53,40	65,60
	Negative	M	8	60,56 ^{A,a}	2,00	57,00	63,20
	Positivo	M	16	59,71 ^{A,a}	1,95	56,50	64,20
CHCM (g/dL)	Negative	F	11	32,68 ^{A,a}	1,25	31,20	35,20
	Positivo	F	30	32,52 ^{A,a}	0,97	31,10	35,00
	Negative	M	9	32,49 ^{A,a}	1,08	30,80	33,80
	Positivo	M	16	32,84 ^{A,a}	1,09	31,00	34,80
Platelets (µL)	Negative	F	11	589909,09 ^{A,a}	235148,66	224000	960000
	Positivo	F	30	584466,67 ^{A,a}	163118,47	284000	943000
	Negative	M	8	509375,00 ^{A,a}	164255,84	255000	721000
	Positivo	M	16	565437,50 ^{A,a}	180473,44	320000	894000
Leukocytes (mm^3)	Negative	F	10	14790,00 ^{A,a}	3682,22	8100	21200
	Positivo	F	29	15534,48 ^{A,a}	4125,90	9000	25900
	Negative	M	8	15650,00 ^{A,a}	6335,84	7500	25500
	Positivo	M	16	15756,25 ^{A,a}	3940,89	8900	23600

3.6.3. Statistical analyses comparing positive x negative animals for *Hepatozoon* regarding blood parameters during all recaptures (PCA)

The PCA resulted in a large overlap of captures (ellipses), which indicates that based on the present data there is little difference in the set of coatis' blood parameters between the different captures, with positive and negative animals for *Hepatozoon* presenting similar values for all blood parameters (Fig. 2). Even though ellipses 5 and 6 are out of the pattern, this finding should be analyzed with caution, considering that few animals were recaptured, leading the estimate of the ellipse to be biased.

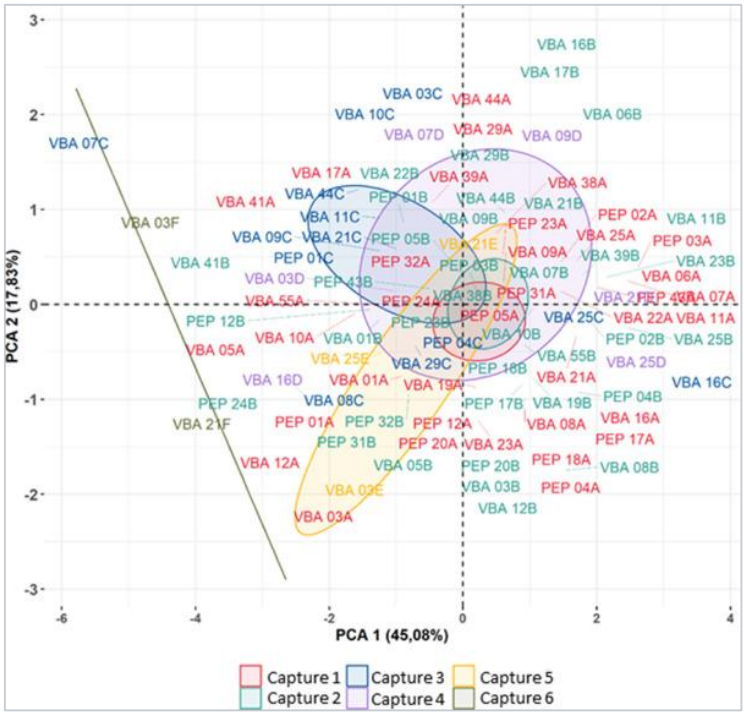

Fig. 2. Scatterplot of the first and second principal components (PCA) of the seven blood parameters and blood smear results (presence or absence of *H. procyonis* gametocytes). Ellipses were grouped by captures and recaptures (capture 1, 2, 3, 4 5 and 6) with 95% confidence.

4. Discussion

The detection of gametocytes by blood smears analyses is a routine diagnostic method for the detection of *Hepatozoon* sp. in several host species. However, the lack of gametocytes in the smears does not indicate the absence of infection, since the intermittent and low parasitemia have been reported in *Hepatozoon* sp. behavior (Baneth and

Weigler, 1997; Eiras et al., 2007). In the present study, 67 (40%) of the 165 blood smears evaluated (from both areas, including recaptures) had *H. procyonis* gametocytes, with parasitemia ranging from 1 (0.2%) - 50 (10%) gametocytes per 500 leukocytes. Herein, the percentage of animals that showed gametocytes in blood smears was higher when compared to the previous studies from Brazil, also showing higher parasitemia (ranging from 0.2% - 10%). Regarding the detection of gametocytes of *H. procyonis* in coatis´ blood smears from Brazil, two previous studies showed parasites in blood smears from coatis that inhabit urbans forest fragments: da Silva et al. (2018) found 9/83 (10.8%) in ring-tailed coatis from cities of São Paulo state, with parasitemia ranging from 0.2% to 1%; and Estevam et al. (2020) recorded gametocytes in 4/151 (2.6%), without information regarding parasitemia.

Furthermore, Sousa et al. (2017) did not find gametocytes in free-living coatis sampled in the Pantanal biome, Mato Grosso do Sul state, although 13/31 (42%) animals were positive in cPCR for *Hepatozoon* sp. based on the 18S rRNA gene. We highlight that anthropogenic pressures may favor higher parasitemia in coatis since our results, together with da Silva et al. (2018) and Estevam et al. (2020), observed parasitemia in coatis that inhabit urban forest fragments and Souza et al. (2017) failed to find gametocytes in blood smears from coatis obtained in natural fragments from Pantanal. These results demonstrate the importance of constant monitorization of the health of the coatis that inhabit urban forest fragments for conservation and one health purposes.

In the present study, among the 50 animals (32 PEP and 18 VBA) that presented gametocytes of *H. procyonis* in blood smears, 23 were male (13 PEP and 10 VBA) and 27 were female (19 PEP and 8 VBA). Also, from 80 positive nPCR animals, 47 were females (20 PEP and 27 VBA) and 33 were males (19 PEP and 14 VBA). Despite a slightly larger number of positive females in blood smears and in nPCR, no statistical differences were observed regarding the occurrence of the parasite in relation to the gender of the sampled animals. Some studies indicate that host characteristics, such as gender, can affect parasitism patterns (Klein, 2004; Monello and Goomper, 2010). The prevalence and intensity of parasitic infections appear to be higher in males than in females. This fact can be explained by the behavior of some species, in which males, more prone to aggressive interactions (to conquer females and territories) and dispersion, would be more likely to have contact with parasites (Klein, 2004).

Furthermore, due to the difference in hormonal levels and fluctuation, females have a greater immune response than males, which can be beneficial in the immune response against parasites (Zuk and Mckean, 1996; Klein, 2000). However, several studies have found

no gender differences in the prevalence or intensity of parasite infection (da Silva et al., 2018, Hokan et al., 2017) and some have even found higher rates of parasite infection in females (Shiddo et al., 1995). In previous study carried out with coatis in the southeastern region of Brazil, da Silva et al. (2018) did not observe statistical differences in the *H. procyonis* parasitemia of male and female coatis blood smears. However, the authors suggested that the results should be considered with caution, given the small number of infected animals (n = 9). Estevam et al. (2020) also did not observe statistical differences in *H. procyonis* parasitemia between male and female coatis, but also had a small number of positive animals.

Previous studies suggested that ring-tailed coatis would have a similar social system to white-nosed coatis. While solitary adult males live apart from the group outside the mating season, females live in groups with their offspring (Gittleman, 1989; Gompper, 1995, 1996; Beisiegel, 2001). This characteristic may lead to higher prevalence and intensity of parasitic infections in males than in females because they tend to disperse in a larger territory (Klein, 2004). However, studies about dietary patterns and behavior of ring-tailed coatis showed different features, with adult males observed living together with females and offspring outside the mating season in five different coati populations in Brazil (Mangabeiras Park, Minas Gerais; Tietê Ecological Park, São Paulo; Nhumirim ranch, Pantanal; *Parque Estadual do Prosa*, Mato Grosso do Sul; and Campeche Island, Santa Catarina) and in Argentina, Foz do Iguaçu (Alves-Costa et al., 2004; Resende et al., 2004; Costa et al., 2009; Hirsch, 2011a,b). It seems that both in natural areas (Olifiers et al., 2009) and anthropized areas (such as those sampled in the present study) males remain associated with the group off mating season. This behavior might explain the lack of statistical differences in the *H. procyonis* parasitemia between males and females in the present and previous studies. Apparently, adult males and females present the same risk of infection with *H. procyonis*, since they share the same territory inside and outside the mating season.

Understanding the parasite-host relationship is essential for investigating the dynamics of the diseases and the evolutionary implications of the parasites in their hosts and ecosystems. A commonly observed pattern in this relationship is a higher parasitic intensity in young animals compared to adult animals (Gregory et al., 1992; Hudson and Dobson, 1997). Reasons for this difference are not fully understood, and may vary according to the parasite species, host and environment. Three non-mutually exclusive hypotheses have been proposed, namely the hypothesis of selection, immunity and exposure to vectors (Sol et al. 2003). The selection theory proposes that young animals with a high parasitic

burden would die before reaching adulthood (Hudson and Dobson, 1997; Martin et al., 2001); the immunity theory is related to the development of host immunity to the parasite, which would consequently reduce the intensity of the parasite in adult hosts; and, finally, the vector exposure theory is related to the differences in animal behavior, which would make adults less exposed to vectors when compared to juveniles, for example, during the search for habitat to establish new territories (Wunderle, 1991; Hudson and Dobson, 1997).

In relation to protozoa of the genus *Hepatozoon*, several studies have been carried out with *H. canis*, but little is known about the parasite-host relationships of other species belonging to this genus. Some studies reported a higher frequency of *H. canis* gametocytes in the blood smears of young animals, which are likely to be in the acute stage of the disease. Mundim et al. (1994) observed that *H. canis* infection was more prevalent in dogs under 1 year of age (59%). Baneth and Weigler (1997) found infected dogs in all studied age groups, whilst the prevalence was higher in dogs under 6 months age and in dogs from 5 to 10 years old. da Silva et al. (2018) and Estevam et al. (2020) did not observe statistical differences in coatis' parasitemia among the sampled age groups. In the present study, among nPCR positive coatis, 19/28 (67%) were immature animals (infant and subadults) and 56/76 (73%) were adults. Although the prevalence seems higher in adults, no statistical differences were observed. Since infants leave the trees nests around 5-8 weeks after birth (Hirsch, 2007; Olifiers et al., 2009), and then remains as subadults in the same group, we can assume that all ages have similar chances to be in contact with arthropod vectors.

Regarding molecular results, Sousa et al. (2017), when testing two cPCR assays based on the 18S rRNA on blood samples from 31 coatis in the Pantanal Sul-matogrossense, found negative results in the protocol described by Perkins and Keller (2001) and 13 (42%) positive samples in the protocol proposed by Ujvari et al. (2004). Phylogenetics analyses grouped the obtained sequences in a large clade composed by *H. canis, H. americanum* and *H. felis* (carnivorous clade). da Silva et al. (2018) firstly submitted 83 coatis blood samples (40 from Palmital, 30 from Botucatu and 13 from São Paulo) to cPCR according to the protocol of Ujvari et al. (2004); the positive samples were submitted to a second reaction (nPCR), also targeting 18S rRNA gene and using a protocol previously described by Mathew et al. (2000) and Criado-Fornelio et al. (2006). Twenty-one (25%) samples were positive for *Hepatozoon* spp., and the obtained sequences were phylogenetically grouped in the large clade of *Hepatozoon* spp. of carnivores. Estevam et al. (2020) submitted the coati's blood samples to a nPCR targeting 18S rRNA of Apicomplexa protozoans (Babesia, Theileria, Hepatozoon and Sarcocystis) (Zahler et al., 2000; Silveira et al., 2011). Out of 216 obtained samples, 101

(47%) were positive in the above-mentioned PCR assays and 69 (32%) showed high identity with *Hepatozoon* species, among *H. americanum* (99%), *Hepatozoon* sp. from Dromiciops gliroides (95-98%), and *Hepatozoon* sp. from Brazilian Pantanal (97%). In the present study, 112/165 (68%) samples were positive in the referred nPCR based on the protocols of Mathew et al. (2000) and Criado-Fornelio et al. (2006) (46 from PEP and 66 from VBA). This study presented a higher detection when comparing the percentage of positive animals from previous studies. This fact might be explained since it was used a nPCR for screening, which is known to be more sensitive than cPCR assays. Also, this high occurrence might be related to the gregarious behavior of this Procyonidae species, as previously observed by de Sousa et al. (2017). The frequent grooming behavior of this mammal species might have favored the ingestion of *H. procyonis*-containing ticks, which may act as definitive hosts for this *Hepatozoon* species.

Even though it is known that vertebrate hosts become infected after ingestion of sporulated oocysts in infected ticks, the vector for *H.* procyonis is yet to be described. At the present study, *Hepatozoon* sp. DNA was detected in pools of *Amblyomma* sp., pools of *A. sculptum* and *A. dubitatum* nymphs and in one *A. sculptum* adult female. No *A. ovale* adult was positive at nPCR assays. Sousa et al. (2017) described infestation by *A. sculptum, Amblyomma parvum* Aragão 1908*, A. ovale, Rhipicephalus (Boophilus) microplus* Canes 1888*,* and larvae of *Amblyomma* spp. in coatis sampled in the Pantanal Sul-Matogrossense. In São Paulo state, *A. dubitatum, A. ovale, A. sculptum, Amblyomma* sp. and *Rhipicephalus sanguineus* sensu lato Latreille 1806 were collected from coatis, with a higher prevalence of *A*. ovale and A. sculptum. Most of the coatis that were positive for H. procyonis were found to be infested by A. ovale and the authors suggested that this tick could act as a vector for H. procyonis (da Silva et al., 2018). Indeed, A. ovale play a role as vector of H. canis in Brazil (Rubini et al., 2009). Estevam et al. (2020) collected a total of 36 ticks from ring-tailed coatis in Mangabeiras Municipal Park, Minas Gerais; two larvae (Amblyomma sp.), 32 nymphs (Amblyomma sp.), and two adults were found in different coatis (one with A. ovale and one with A. sculptum, both female). In the present study, coatis were infested by *Amblyomma* sp. larvae, *A. sculptum, A. ovale,* and *A. dubitatum*, with a higher prevalence of *A. sculptum* and *A. dubitatum*. Although *A. sculptum* seems to show little or no importance in the canine hepatozoonosis epidemiology (Demoner et al., 2013, 2016), this tick species might have importance on *H. procyonis* transmission, since it was collected in all areas with the presence of positive coatis from previous works and in the present study. Although *Hepatozoon* DNA was detected in ticks, it is not possible to assume/suggest if these tick species play a role as *H. procyonis* vector,

since *Hepatozoon* DNA may represent the remnant of the feed blood from vertebrate hosts. Future experimental studies should be conducted in order to check the vector competence of *Amblyomma* species in the transmission of *H. procyonis* to ring-tailed coatis.

Many studies performed in Brazil have shown apparently healthy dogs infected with *H. canis* (O'Dwyer et al., 2001, Rubini et al., 2008; Spolidorio et al., 2009). Even though *Hepatozoon* species are considered opportunist parasites, and are usually associated with other pathogens, a small number of *H. canis*-infected dogs in Brazil were reported showing clinical/hematological abnormalities, such as apathy, anorexia, pale mucosal, progressive weight loss and anemia and leukopenia (Da Silva et al., 2011; Paiz et al., 2016). No study regarding health parameters (clinical examination and hematological parameters) has been performed in coatis infected with *H. procyonis* in Brazil to date. Among the sampled coatis in the present study, skin lesions were the most frequent physical abnormality found, and they are more likely to be related to fights or the capture procedure than caused by the parasitism by *H. procyonis*. Also, few animals had alteration of the membrane mucous coloration; indeed, coatis with the highest parasitemia presented normal coloration of mucous membrane. Regarding hematological parameters, it seems that the presence of *H. procyonis* does not cause significant alterations, since no statistical difference was observed when comparing infected x non-infected animals. On the other hand, anemia and leukocytosis are the main hematological alteration in dogs parasitized by *H. canis* (Paiz et al., 2016). Herein, animals with the highest *H. procyonis* parasitemia did not present significant alterations in erythrocyte and leucocyte counts. Finally, we cannot rule out the occurrence of coinfection with other vector-borne pathogens, which might have caused hematological and clinical abnormalities. Also, no factor showed significant difference ($p<0.05$) in the binomial logistic regression model when comparing different ages and genders, indicating that the difference in the proportion of *H. procyonis*-positive ring-tailed coatis within each factor (i.e. M and F) was a result of chance. The confidence interval was very wide (CIsup – ICinf) for all factors. One suggestion to reduce this CI and, consequently, potentially visualize an association between nPCR-positive animals and the studied factors would be to increase the sample size, which sometimes is not simple to achieve when working with free-living animals.

5. Conclusions

Hepatozoon procyonis was found parasitizing coatis in two sampled areas (PEP and VBA) in Campo Grande, Mato Grosso do Sul state, central-western Brazil, using both blood

smears and 18S rRNA based-molecular assay (nPCR). Overall, 77% of animals were positive for *H. procyonis* in at least one capture. *H. procyonis* infection was verified in coatis of all age (infants, subadults and adults), gender groups (male and female), in both areas (PEP and VBA) and at rainy and dry periods of the year, with no statistical differences among the groups. Fluctuation of parasitemia (number of *H. procyonis* gametocytes/500 leukocytes) throughout the recaptures was observed, a finding already observed in dogs parasitized by *H. canis. Hepatozoon* DNA was detected in *Amblyomma* larvae, *A. dubitatum* and *A. scultpum* nymphs and in one *A. sculptum* adult. *H. procyonis* infection seems not to significantly influence clinical and hematological parameters of the sampled coatis.

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CRediT authorship contribution statement

L. Perles: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. G.C. de Macedo: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. W.T.G. Barreto: Data curation, Investigation, Methodology, Writing – review & editing. G.V. Francisco: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. H.M. Herrera: Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing. D.M Barros-Battesti: Data curation, Investigation, Methodology, Writing – review & editing. R.Z. Machado: Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. M.R. André: Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of interest

The authors declare that they have no conflict of interest.

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Supplementary file

Longitudinal dynamics and health impact of *Hepatozoon procyonis* (Apicomplexa: Hepatozoidae) in naturally infected ring-tailed coatis *Nasua nasua* (Carnivora: Procyonidae)

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Table 1. Sampling data, identification number, sex, age, weight and parasitemia in blood smears of coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do sul state, Brazil, between march 2018 and april 2019.

Sampling data	Identification number	Sex	Age	Weight (gr)	Parasitemia in blood smears
30/05/2018	PEP 01B	M	Adult	5580	Negative
08/08/2018	PEP 01C	M	Adult	4365	Negative
08/10/2018	PEP 01D	M	Adult	4540	8/500 (1,6%)
13/03/2018	PEP 02	M	Adult	3230	2/500 (0,4%)
30/05/2018	PEP 02B	M	Adult	5915	8/500 (1,6%)
30/05/2018	PEP 03B	F	Adult	4145	2/500 (0,4%)
31/07/2018	PEP 03C	F	Adult	2840	31/500 (6,2%)
14/03/2018	PEP 04	M	Adult	5110	23/500 (4,6%)
15/06/2018	PEP 04B	M	Adult	4770	1/500 (0,2%)
30/07/2018	PEP 04C	M	Adult	4930	5/500 (1%)
30/05/2018	PEP 05B	M	Adult	7335	3/500 (0,6%)
01/08/2018	PEP 05C	M	Adult	3290	Negative
15/03/2018	PEP 06	F	Adult	4545	3/500 (0,6%)
15/03/2018	PEP 08	F	Adult	6845	4/500 (0,8%)
16/03/2018	PEP 10	M	Adult	6600	3/500 (0,6%)
16/03/2018	PEP 11	F	Adult	7970	8/500 (1,6%)
11/06/2018	PEP 12B	F	Adult	4090	11/500 (2,2%)
06/08/2018	PEP 12C	F	Adult	4790	16/500 (3,2%)
20/03/2018	PEP 13	M	Subadult	4285	9/500 (1,8%)
21/03/2018	PEP 14	M	Adult	5730	Negative
21/03/2018	PEP 15	F	Adult	3855	Negative
22/03/2018	PEP 17	F	Adult	6870	Negative
15/06/2018	PEP 17B	F	Adult	2105	7/500 (1,4%)
28/05/2018	PEP 18	F	Cub	1970	Negative
01/10/2018	PEP 18B	F	Cub	3740	Negative
28/05/2018	PEP 19	F	Cub	4545	Negative
29/05/2018	PEP 20	F	Adult	4530	6/500 (1,2%)
31/07/2018	PEP 20B	F	Adult	6020	39/500 (7,8%)

29/05/2018	PEP 21	F	Adult	5530	3/500 (0,6%)
29/05/2018	PEP 22	F	Adult	4170	1/500 (0,2%)
30/05/2018	PEP 23	F	Adult	5240	1/500 (0,2%)
30/07/2018	PEP 23B	F	Adult	4315	1/500 (0,2%)
12/06/2018	PEP 24	F	Adult	3770	Negative
02/08/2018	PEP 24B	F	Adult	4105	9/500 (1,8%)
12/06/2018	PEP 25	M	Adult	4780	3/500 (0,6%)
09/08/2018	PEP 26B	M	Adult	4645	2/500 (0,4%)
31/07/2018	PEP 27	F	Adult	3900	Negative
31/07/2018	PEP 28	M	Adult	2820	Negative
31/07/2018	PEP 29	F	Adult	3695	40/500 (8%)
31/07/2018	PEP 30	F	Adult	4080	1/500 (0,2%)
01/08/2018	PEP 31	M	Adult	4020	3/500 (0,6%)
03/10/2018	PEP 31B	M	Adult	3765	18/500 (3,6%)
02/08/2018	PEP 32	M	Adult	3935	Negative
04/10/2018	PEP 32B	M	Adult	3685	Negative
02/08/2018	PEP 33	M	Adult	4600	Negative
03/08/2018	PEP 34	F	Adult	NR	Negative
03/08/2018	PEP 35	F	Adult	4065	2/500 (0,4%)
03/08/2018	PEP 36	F	Subadult	7350	1/500 (0,2%)
06/08/2018	PEP 37	F	Adult	1670	Negative
06/08/2018	PEP 38	F	Adult	7615	Negative
07/08/2018	PEP 39	M	Adult	1420	Negative
08/08/2018	PEP 40	F	Adult	3705	50/500 (10%)
08/08/2018	PEP 42	F	Adult	4450	1/500 (0,2%)
09/08/2018	PEP 43	M	Adult	4275	2/500 (0,4%)
09/10/2018	PEP 43B	M	Adult	1600	2/500 (0,4%)
02/10/2018	PEP 44	M	Cub	4105	Negative
02/10/2018	PEP 45	M	Cub	3800	Negative
02/10/2018	PEP 46	F	Cub	4450	Negative
02/10/2018	PEP 47	M	Cub	4850	16/500 (3,2%)
04/10/2018	PEP 48	F	Adult	5890	1/500 (0,2%)

04/10/2018	PEP 49	M	Cub	3830	7/500 (1,4%)
09/10/2018	PEP 50	F	Adult	4365	1/500 (0,2%)
10/10/2018	PEP 51	M	Adult	3725	5/500 (1%)
30/04/2018	VBA 01	M	Adult	3520	Negative
26/04/2019	VBA 01B	M	Adult	5810	2/500 (0,4%)
02/05/2018	VBA 03	F	Adult	4720	Negative
18/06/2018	VBA 03B	F	Adult	4210	Negative
28/08/2018	VBA 03C	F	Adult	6010	Negative
23/10/2018	VBA 03D	F	Adult	5500	Negative
23/01/2019	VBA 03E	F	Adult	4045	Negative
20/03/2019	VBA 03F	F	Adult	3985	1/500 (0,2%)
02/05/2018	VBA 04	F	Adult	3365	Negative
02/05/2018	VBA 05	M	Adult	3200	Negative
23/01/2019	VBA 05D	M	Adult	3645	Negative
02/05/2018	VBA 06	M	Adult	4260	Negative
07/11/2018	VBA 06B	M	Adult	7245	Negative
03/05/2018	VBA 07	M	Adult	4580	3/500 (0,6%)
25/06/2018	VBA 07B	M	Adult	3400	Negative
21/08/2018	VBA 07C	M	Adult	4800	Negative
22/10/2018	VBA 07D	M	Adult	4080	3/500 (0,6%)
04/05/2018	VBA 08	F	Adult	4430	Negative
19/06/2016	VBA 08B	F	Adult	3370	Negative
28/03/2019	VBA 08C	F	Adult	4795	Negative
04/05/2018	VBA 09	M	Subadult	5145	Negative
26/06/2018	VBA 09B	M	Subadult	3815	Negative
30/08/2018	VBA 09C	M	Adult	3800	Negative
23/10/2018	VBA 09D	M	Adult	1915	Negative
04/05/2018	VBA 10	F	Adult	1900	Negative
20/06/2018	VBA 10B	F	Adult	1655	Negative
20/08/2018	VBA 10C	F	Adult	3270	Negative
07/05/2018	VBA 11	M	Adult	4265	Negative
20/06/2018	VBA 11B	M	Adult	1680	Negative

23/10/2018	VBA 11C	M	Adult	3695	Negative
09/05/2018	VBA 12	F	Adult	3990	Negative
19/06/2016	VBA 12B	F	Adult	3815	Negative
10/05/2018	VBA 13	M	Adult	1915	Negative
19/06/2016	VBA 15	F	Adult	2685	Negative
20/06/2018	VBA 16	F	Adult	3425	Negative
24/08/2018	VBA 16B	F	Adult	2200	Negative
21/01/2019	VBA 16C	F	Adult	1870	Negative
26/03/2019	VBA 16D	F	Adult	3750	Negative
20/06/2018	VBA 17	F	Adult	3270	Negative
29/01/2019	VBA 17B	F	Adult	2660	3/500 (0,6%)
21/06/2018	VBA 18	F	Adult	2545	Negative
21/06/2018	VBA 19	F	Adult	2765	Negative
23/04/2019	VBA 19B	F	Adult	2820	1/500 (0,2%)
22/06/2018	VBA 20	M	Adult	3080	Negative
25/06/2018	VBA 21	M	Cub	5640	2/500 (0,4%)
24/08/2018	VBA 21B	M	Cub	3675	3/500 (0,6%) extracelular gamonts
29/10/2018	VBA 21C	M	Subadult	5080	Negative
29/01/2019	VBA 21D	M	Subadult	2530	24/500 (4,8%)
21/03/2019	VBA 21E	M	Subadult	5260	11/500 (2,2%)
23/04/2019	VBA 21F	M	Subadult	3800	2/200 (0,4%)
26/06/2018	VBA 22	F	Cub	3825	Negative
30/08/2018	VBA 22B	F	Cub	4685	Negative
26/06/2018	VBA 23	F	Adult	5380	Negative
31/10/2018	VBA 23B	F	Adult	3960	Negative
26/06/2018	VBA 24	M	Subadult	4125	Negative
26/06/2018	VBA 25	M	Cub	2665	Negative
27/08/2018	VBA 25B	M	Cub	3075	Negative
06/11/2018	VBA 25C	M	Cub	2870	25/500 (5%)
29/01/2019	VBA 25D	M	Subadult	2805	1/500 (0,2%)

27/03/2019	VBA 25E	M	Subadult	2540	23/500 (4,6%)
29/06/2018	VBA 26	F	Adult	3460	Negative
20/08/2018	VBA 27	M	Adult	2670	1/500 (0,2%)
24/08/2018	VBA 28	F	Adult	2340	Negative
24/08/2018	VBA 29	M	Cub	5455	Negative
31/10/2018	VBA 29B	M	Cub	4780	Negative
22/01/2019	VBA 29C	M	Subadult	4990	Negative
24/08/2018	VBA 30	F	Cub	3470	Negative
27/08/2018	VBA 31	F	Adult	2970	Negative
27/08/2018	VBA 32	F	Adult	3735	Negative
28/08/2018	VBA 33	F	Adult	3040	Negative
28/08/2018	VBA 34	M	Subadult	3530	Negative
28/08/2018	VBA 35	M	Subadult	2470	Negative
28/08/2018	VBA 36	M	Cub	3600	Negative
29/08/2018	VBA 37	F	Adult	3670	6/500 (1,2%)
30/08/2018	VBA 38	F	Cub	3250	5/500 (1%)
25/03/2019	VBA 38B	F	Subadult	3470	1/500 (0,2%)
31/10/2018	VBA 39B	F	Adultt	6830	Negative
21/01/2019	VBA 39C	F	Adult	3675	1/500 (0,2%)
31/08/2018	VBA 40	F	Adult	3815	2/500 (0,4%)
01/11/2018	VBA 41	F	Adult	4275	Negative
21/01/2019	VBA 41B	F	Adult	2820	Negative
06/11/2018	VBA 42	M	Cub	3600	3/500 (0,6%)
06/11/2018	VBA 43	M	Adult	3665	Negative
07/11/2018	VBA 44	M	Adult	4650	Negative
21/03/2019	VBA 44B	M	Adult	4135	Negative
30/04/2019	VBA 44C	M	Adult	6590	Negative
22/01/2019	VBA 45	F	Adult	4355	Negative
22/01/2019	VBA 46	F	Cub	7455	Negative
23/01/2019	VBA 47	F	Adult	4285	Negative
23/01/2019	VBA 48	F	Adult	2890	Negative
23/01/2019	VBA 49	F	Adult	4730	Negative

29/01/2019	VBA 50	M	Cub	4035	11/500 (2,2%)
30/01/2019	VBA 52	F	Subadult	3985	5/500 (1%)
21/03/2019	VBA 53	F	Adult	3700	Negative
21/03/2019	VBA 54	M	Subadult	1700	2/500 (0,4%)
22/03/2019	VBA 55	M	Adult	4750	Negative
30/04/2019	VBA 55B	M	Adult	7440	Negative
25/03/2019	VBA 56	F	Subadult	4775	Negative
25/03/2019	VBA 57	F	Adult	4015	Negative
23/04/2019	VBA 58	M	Cub	7140	Negative
23/04/2019	VBA 59	M	Adult	6535	2/200 (0,4%)
25/04/2019	VBA 60	M	Subadult	7260	8/500(1,6%)

Table 2. Descriptive analyses and *Odds Ratio* comparing nPCR positive vs. negative coatis for 18SrRNA of *Hepatozoon procyonis*.

Variable		***Hepatozoon* at nPCR**		**OR**	**ICinf**	**ICsup**	***p-valor***
		Positive	**Negative**				
Season	Rainy	20	8				0,519
	Dry	27	12	0,67	0,20	2,30	
Locality	PEP	22	11				0,424
	VBA	25	9	1,55	0,52	4,60	
Sex	F	29	11				0,679
	M	18	9	0,79	0,27	2,34	
Age	Immature	14	8				0,311
	Mature	33	12	1,89	0,55	6,47	

OR: *Odds Ratio* ; **ICinf**: Inferior confidence interval; **ICsup**: Superior confidence interval; **F**: Female; **M**: Male; **PEP**: Parque Estadual do Prosa; **VBA**: Vila da Base Aérea

Table 3. Descriptive analyses of hematological parameters comparing positive vs. negative coatis for 18SrRNA of *Hepatozoon procyonis*.

Variable	PCR	Sex	n	Men	SD	Min	Max
Red Blood cells (RBC (10^6/µL)	Negative	F	10	5,24 ^{A,a}	0,22	4,79	5,62
	Positivo	F	29	5,29 ^{A,a}	0,40	4,42	6,04
	Negative	M	9	5,51 ^{A,b}	0,40	5,08	6,15
	Positivo	M	16	5,66 ^{A,b}	0,46	4,90	6,44
Hemoglobina (g/dL)	Negative	F	11	10,05 ^{A,b}	0,60	9,00	11,20
	Positivo	F	30	10,23 ^{A,b}	0,91	8,40	12,00
	Negative	M	9	10,70 ^{A,b}	0,73	9,50	11,60
	Positivo	M	16	11,11 ^{A,b}	1,21	9,50	13,30
Hematocrit (%)	Negative	F	9	30,81 ^{A,b}	1,28	28,40	33,20
	Positivo	F	30	31,42 ^{A,b}	2,29	26,10	36,40
	Negative	M	9	32,89 ^{A,b}	1,35	30,80	34,70
	Positivo	M	16	33,77 ^{A,b}	2,96	29,10	38,20
MCV (fL)	Negative	F	10	58,40 ^{A,a}	2,46	54,30	63,20
	Positivo	F	29	59,97 ^{A,a}	2,72	53,40	65,60
	Negative	M	8	60,56 ^{A,a}	2,00	57,00	63,20
	Positivo	M	16	59,71 ^{A,a}	1,95	56,50	64,20
CHCM (g/dL)	Negative	F	11	32,68 ^{A,a}	1,25	31,20	35,20
	Positivo	F	30	32,52 ^{A,a}	0,97	31,10	35,00
	Negative	M	9	32,49 ^{A,a}	1,08	30,80	33,80
	Positivo	M	16	32,84 ^{A,a}	1,09	31,00	34,80
Platelets (µL)	Negative	F	11	589909,09 ^{A,a}	235148,66	224000	960000
	Positivo	F	30	584466,67 ^{A,a}	163118,47	284000	943000
	Negative	M	8	509375,00 ^{A,a}	164255,84	255000	721000
	Positivo	M	16	565437,50 ^{A,a}	180473,44	320000	894000
Leukocytes (mm^3)	Negative	F	10	14790,00 ^{A,a}	3682,22	8100	21200
	Positivo	F	29	15534,48 ^{A,a}	4125,90	9000	25900
	Negative	M	8	15650,00 ^{A,a}	6335,84	7500	25500
	Positivo	M	16	15756,25 ^{A,a}	3940,89	8900	23600

n: number of coatis evaluated; **SD:** standard deviation; **Min**: minimum; **Max**: maximum; Capital letters indicate significant Statistical difference ($p<0,05$) between positive vs. Negative coatis at nPCR; Lower case letters indicate significant Statistical difference ($p<0,05$) between female vs. male.

Capítulo 5* – Multi-locus sequencing reveals putative novel Anaplasmataceae agents, '*Candidatus* Ehrlichia dumleri' and *Anaplasma* sp., in ring-tailed coatis (*Nasua nasua*) from Midwestern Brazil

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Abstract: The Anaplasmataceae family encompasses obligate intracellular α-proteobacteria of human and veterinary medicine importance. This study performed multi-locus sequencing to characterize *Ehrlichia* and *Anaplasma* in coati's blood samples in Midwestern Brazil. Twenty-five samples (25/165—15.1%) were positive in the screening PCR based on the *dsb* gene of *Ehrlichia* spp. and were characterized using 16S rRNA, *sodB*, *groEL*, and *gltA* genes and the 23S-5S intergenic space region (ITS). Phylogenetic analyses based on all six molecular markers positioned the sequences into a new clade, with a common origin of *Ehrlichia ruminantium*. Haplotype analyses of 16S RNA sequences revealed the presence of two distinct *Ehrlichia* genotypes. Six samples (6/165, 3.6%) were positive in the screening nPCR for the 16S rRNA gene of *Anaplasma* spp. and were submitted to an additional PCR targeting the ITS for molecular characterization. Phylogenetic analyses based on both 16S rRNA gene and ITS positioned the *Anaplasma* sp. detected in the present study in a large clade with other *Anaplasma* sp. previously detected in ticks and wild animals and in a clade with '*Candidatus* Anaplasma brasiliensis', respectively. Based on distinct molecular markers, the present work described a putative novel Anaplasmataceae agent, namely '*Candidatus* Ehrlichia dumleri', and *Anaplasma* sp. closely related to the previously described '*Candidatus* Anaplasma brasiliensis'.

Keywords: rickettsiales; procyonidae; tick-borne agents; wild animals

1. Introduction

The Anaplasmataceae family (Order Rickettsiales) encompasses obligate intracellular α-proteobacteria that can cause important diseases in humans and animals [1,2]. Wild and domestic carnivores are considered an important source of tick-borne agents, especially in

anthropized areas, where close contact between domestic/wild animals and humans can occur. Procyonidae mammals, including ring-tailed coatis *Nasua nasua* from Brazil, can easily adapt to peri-urban areas [3,4], favoring the exchange of ectoparasites and vector-borne pathogens among humans and animals. In the United States, raccoons (*Procyon lotor*) are incriminated as important reservoirs of *Anaplasma phagocytophilum,* a zoonotic agent transmitted by ticks from *Ixodes persulcatus* complex [1,5–7]. More recently, an unusual case of human anaplasmosis was reported in the Amazon, French Guiana, caused by '*Candidatus* Anaplasma sparouinense', a putative novel *Anaplasma* sp. closely related to *Anaplasma* genotypes previously detected in *Amblyomma coelebs* ticks collected from coatis, rats, and sloths ('*Candidatus* Anaplasma amazonensis') [2].

Although coatis from urban forest fragments at Campo Grande city, Mato Grosso do Sul state, Brazil seem to present tick species diversity lower than those from natural areas, *Amblyomma dubitatum*, *Amblyomma sculptum*, and *Amblyomma ovale* were identified parasitizing this gregarious species in [8]. In fact, coatis can be found infected with *Trypanosoma cruzi* and *Leishmania* spp., two zoonoses of extreme importance in Public Health [9,10]. These findings demonstrate the importance of constant surveillance on vector-borne agents in wild fauna, especially those with zoonotic potential. The present study aimed to detect the presence of Ehrlichia and Anaplasma spp. in coati's blood samples molecularly and assess the phylogenetic positioning of the detected agents based on a multi-locus sequencing approach.

2. Materials and Methods

2.1. Ethical Statement

All experimental procedures were approved by the 'Instituto Chico Mendes de Biodiversidade' (ICMBio) (SISBIO 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB 001/2018) and Air Force Cooperation Agreement (Nº01/GAP-CG/2018).

2.2. Blood Sampling

Between March 2018 and January 2019, coatis (*N. nasua*) were sampled every three months for 10 consecutive nights (with an interval of one day—Saturday) in two Cerrado areas located in Campo Grande city, Mato Grosso do Sul State, Midwestern Brazil. All captures and recaptures were performed by convenience, and recapture times were performed by chance. The first sampling spot, *Parque Estadual do Prosa* (PEP) (−20.44987,

−54.56529) is a conservation unit of 135 hectares. The second sampling spot was a Brazilian Air Force Private Area (VBA) (−20.47163, −54.65405), a complex of 197 hectares divided into a military operational area and a residential area inhabited by at least 730 humans with domestic animals such as dogs.

All capture procedures were previously described [4,8,11]. After chemical restraint, animals were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue between the shoulder blades. They were measured, and the age was estimated according to the reference literature [12]. Blood was sampled from the femoral vein using tubes containing EDTA (Ethylenediamine tetraacetic acid) and then placed into RNAse/DNAse free cryotubes and stored in an −80 °C freezer until molecular analyses.

2.3. DNA Extraction from Coatis' Blood Samples and Conventional PCR (PCR) for Mammalian Endogenous Control

DNA was extracted from 200 µL of blood using Illustra Blood Mini Kit (GE Healthcare®, Chicago, IL, USA), according to the manufacturer's instructions. To evaluate the quality of the extracted DNA and avoid false negative results, DNA samples were tested by a PCR targeting the mammalian *gapdh* [13]. Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in PCR assays.

2.4. Screening and Characterization of Anaplasmataceae Agents

Screening for *Ehrlichia* spp. was performed using a PCR targeting the *dsb* gene (~409 bp) [14]. Positive samples were submitted to the following PCR or nested PCR (nPCR) protocols for additional molecular characterization: 16S rRNA (~1470 bp) [15], *groEL* (~680 bp) [16], *sodB* (~ 600 bp) [17], *gltA* (~800 bp) [18] and ITS 23S–5S (~300 bp) [19]. Screening for *Anaplasma* spp. was performed using an nPCR targeting the 16S rRNA gene (~546 bp) [20]. Positive samples were submitted to PCR protocols for additional molecular characterization targeting the ITS 23S–5S (~300 bp) [19] and *groEL* (~340 bp) [21]. The PCR reactions contained 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1 mM $MgCl_2$ (Life Technologies®, Carlsbad, CA, USA), 0·2 mM deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies®, Carlsbad, CA, USA), 1·5 U Taq DNA Polymerase (Life Technologies®, Carlsbad, CA, USA), and 0·5 µM of each primer (Integrated DNA Technologies®, Coralville, IA, USA) (Table S1). *Ehrlichia canis* (Jaboticabal strain) and *Anaplasma phagocytophilum* (Webster strain) DNA were used as positive controls. Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in all PCR assays.

The amplicons obtained from PCR and nPCR assays were subjected to 1% ethidium bromide-stained agarose gel electrophoresis and results were visualized in UV transilluminator (ChemiDoc MP Imaging System, Bio Rad®, Hercules, CA, USA). Amplicons that presented high intensity on agarose gel (size of the amplicon matching the control, unique and strong bands) were purified using Exosap Life Technologies®, Carlsbad, CA, USA) and subjected to sequencing. The sequencing of amplicons was realized by the Sanger method [22] using ABI PRISM 3730 DNA Analyzer (Applied Biosystems) at Human Genome and Stem Cell Research Center, 'Instituto de Biociências', University of São Paulo (USP), São Paulo, SP, Brazil. All obtained sequences were submitted to GenBank under the following accession numbers: *Ehrlichia* sp. *dsb* (OP819944–OP819946), *Ehrlichia* sp. 16S rRNA (OM530509-OM530517), *Ehrlichia* sp. *groEL* (OP819939), *Ehrlichia* sp. *sodB* (OP903236-OP903238), *Ehrlichia* sp. 23S-5S (OM717254-OM717255), *Ehrlichia* sp. *gltA* (OP819935-OP919938, OP819940-OP819941), *Anaplasma* sp. 16S rRNA (OP279731-OP279734), and *Anaplasma* sp. 23S-5S (OP918134).

2.5. Bioinformatic Analyses

Electropherograms were submitted to a quality-screening test using Phred-Phrap software (version 23) [23,24] to obtain consensus sequences from the alignment of sense and antisense sequences. BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 4 October 2022) [25] was used to compare the obtained sequences with those previously deposited in the GenBank database to obtain Query-coverage%, E-value, and % identity. For phylogenetic inferences, sequences from the present study were aligned with those retrieved from GenBank using MAFFT software version 7 [26]. The best evolutionary model was chosen using the iqTREE software (available at: http://iqtree.cibiv.univie.ac.at/ accessed on 4 October 2022), under the Akaike Information Criterion (AIC) [27,28]. The phylogenetic analyses were based on Bayesian inference (BI) and performed using MrBayes version 3.1.2 [29] via the CIPRES Science Gateway. Markov chain Monte Carlo simulations were run for 10^6 generations with a sampling frequency of every 100 generations and a 25% burn-in. The phylogenetic tree edition and rooting (outgroup) were performed using TreeGraph 2.0 beta software [30].

Additionally, an analysis of nucleotide polymorphisms of the sequences obtained in the present study was performed. The number of haplotypes, haplotype diversity (Hd), and nucleotide diversity (Pi) were determined using the program DnaSP 5, version 5.10.01 [31]. Also,

the pairwise distance matrix among sequences detected at the present study and other Anaplasmataceae species was estimated using the Mega-X software version 10.1.8 [32].

3. Results

In total, 165 blood samples were collected (63 in PEP and 102 in VBA). In PEP, blood samples were obtained from 47 different individuals (27 females and 20 males; seven infants, 2 subadults and 38 adults). In VBA, samples were obtained from 57 different individuals (32 females and 25 males; 11 infants, 8 sub-adults, and 38 adults) (Table 1). All recapture procedures are previously described [11].

3.1. PCR Assays for Ehrlichia spp. Screening and Characterization

All DNA samples obtained from coati blood (n = 165) were positive in the PCR targeting the *gapdh* endogenous gene. Out of 165 coatis' blood samples, 25 (15.1%) were positive in the screening PCR for the *dsb* gene of *Ehrlichia* spp. [4/63 samples—6.3% from PEP area (from 4 different coatis) and 21/102 samples—20.6% from VBA (obtained from 16 different coatis). No recaptured animal showed positivity in more than one collection time at PEP, while coatis from VBA showed positivity in more than one recapture (Table 1).

Table 1. Identification of ring-tailed coatis (*Nasua nasua*) sampled and recaptured in two areas, namely Parque Estadual do Prosa and Vila da Base Aérea, city of Campo Grande, State of Mato Grosso do Sul, Midwestern Brazil, with the identification number, sex, age and positivity in the screening PCR to *Ehrlichia* sp. (*dsb* gene) during subsequent recaptures.

				Captures					
ID	**Sex**	**Age**	**Local**	**1° Sampling**	**2° Sampling**	**3° Sampling**	**4° Sampling**	**5° Sampling**	**6° Sampling**
PEP05	M [1]	A [3]	PEP [6]	Negative	Positive	Negative	NR	NR	NR
PEP32	M	A	PEP	Negative	Positive	NR	NR	NR	NR
PEP43	M	A	PEP	Negative	Positive	NR	NR	NR	NR
PEP49	M	I	PEP	Positive	NR	NR	NR	NR	NR
VBA01	M	A	VBA [7]	Positive	Negative	NR	NR	NR	NR
VBA03	F[2]	A	VBA	Negative	Positive	Positive	Negative	Positive	Positive
VBA04	F	A	VBA	Positive	NR	NR	NR	NR	NR
VBA08	F	A	VBA	Negative	Negative	Positive	NR	NR	NR
VBA09	M	SA [4]	VBA	Positive	Negative	Negative	Negative	NR	NR
VBA11	M	A	VBA	Negative	Negative	Positive	NR	NR	NR
VBA16	F	A	VBA	Negative	Negative	Negative	Positive	NR	NR
VBA19	F	A	VBA	Positive	Negative	NR	NR	NR	NR
VBA21	M	I [5]	VBA	Negative	Negative	Negative	Negative	Positive	Positive
VBA23	F	A	VBA	Positive	Negative	NR	NR	NR	NR
VBA29	M	F	VBA	Negative	Positive	NR	NR	NR	NR
VBA32	F	A	VBA	Positive	NR	NR	NR	NR	NR
VBA34	M	A	VBA	Positive	NR	NR	NR	NR	NR
VBA43	M	A	VBA	Positive	NR	NR	NR	NR	NR
VBA44	M	A	VBA	Positive	Positive	Positive	NR	NR	NR
VBA57	F	A	VBA	Positive	NR	NR	NR	NR	NR

[1]. male; [2]. female; [3]. adult; [4]. sub-adult [5]. infant; [6]. Parque Estadual do Prosa; [7]. Vila da Base Aérea; NR. Non-recaptured animal.

Transiently positive PCR results were detected in three animals from VBA: VBA03 was positive in four out of six samplings; VBA21 was positive in 2/6 samplings, and VBA44 in 3/3 samplings. No animal from PEP showed PCR positive results for more than one sampling (Table 2). Animal VBA03 remained PCR positive after 276 days after the first positive PCR (18 June 2018–20 March 2019); animal VBA21 after 28 days (21 March 2019–23 April 2019); and VBA44 after 174 days (7 November 2018–30 April 2019).

Table 2. Identification of sampling date (day, month, and year) during subsequent recaptures of ring-tailed coatis (*Nasua nasua*) positive to *Ehrlichia* sp. sampled and recaptured in two areas, namely Parque Estadual do Prosa and Vila da Base Aérea, city of Campo Grande, State of Mato Grosso do Sul, Midwestern Brazil. All dates when positive PCR results were found are bolded.

	1° Sampling	**2° Sampling**	**3° sampling**	**4° Sampling**	**5° Sampling**	**6° Sampling**
VBA03	02 May 2018	18 June 2018	28 August 2018	23 October 2018	23 January 2019	20 March 2019
VBA21	25 June 2018	24 August 2018	29 October 2018	21 January 2019	21 March 2019	23 April 2019
VBA44	07 November 2018	21 March 2019	30 April 2019	NR	NR	NR

NR. non-recaptured animal.

Out of 25 positive samples in the *dsb*-based PCR screening for *Ehrlichia* spp., three were properly sequenced, nine were positive in the PCR targeting the near-complete 16S rRNA gene, one for *groEL* gene, three for *sodB* gene, two for 23S–5S ITS, and six for *gltA* gene. Results from Blast analyses, including sequence size, query coverage, E-value, and % of identity, are shown in Table S1—Supplementary Materials.

Phylogenetic analysis inferred by the Bayesian method (BI) and TIM2+I evolutionary model and based on the *dsb* gene (alignment of 395 bp) grouped the three sequenced samples in a new clade separated (100% posterior probability) from other *Ehrlichia* genotypes previously detected in wild animals from Brazil and *Amblyomma* ticks from Brazil and Argentina (Figure 1). The genetic divergence between sequences obtained in the present study and those closely related and retrieved from GenBank ranged from 0.05 to 0.18 (Supplementary Materials—Table S2).

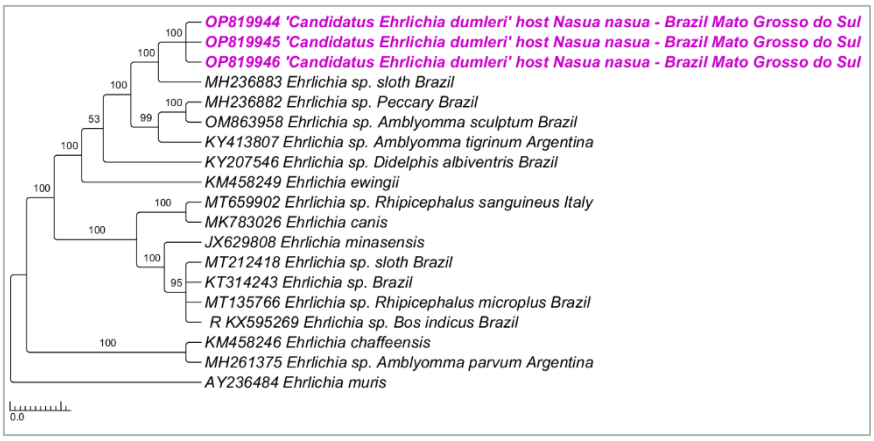

Figure 1. Phylogenetic tree inferred by the Bayesian method and based on the *dsb* gene from *Ehrlichia* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil. Sequences detected in the present study are highlighted in bold pink (VBA).

Phylogenetic analysis inferred by the Bayesian method (BI) and HKY+I+G evolutionary model and based on the 16S rRNA gene (alignment of 1201 bp) grouped the sequences detected in the present study in a new large clade (92% posterior probability) (Figure 2). The genetic divergence between the *Ehrlichia* genotypes detected in coatis from PEP and '*Candidatus* Ehrlichia occidentalis' and *Ehrlichia* sp. *Eira barbara* ranged from 1.2 to 1.7% (Supplementary File—Table S3).

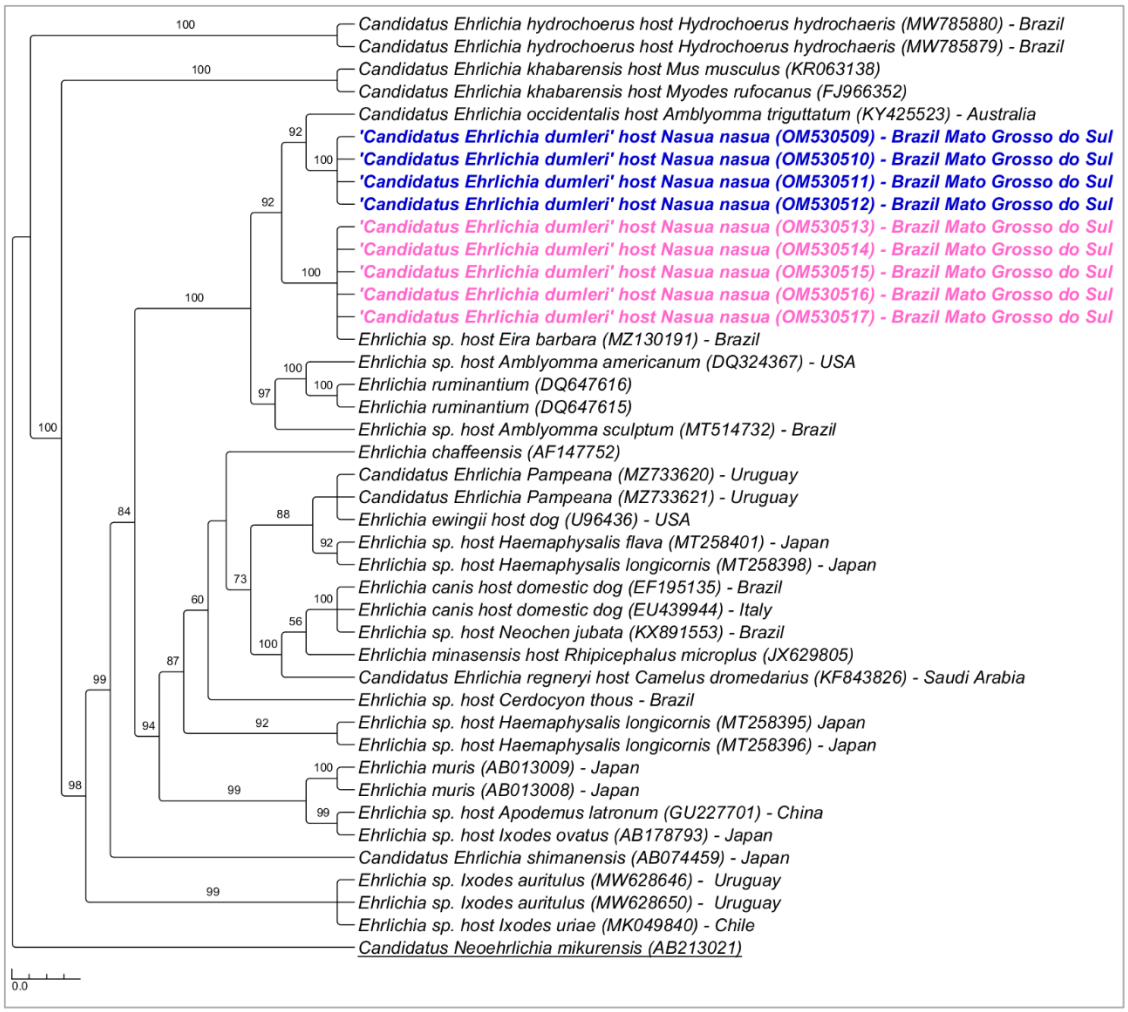

Figure 2. Phylogenetic tree inferred by the Bayesian method and based on the 16S rRNA gene from *Ehrlichia* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil. Sequences detected in the present study are highlighted in bold blue (PEP—genotype 1) and pink (VBA—genotype 2).

Phylogenetic analyses inferred by the Bayesian method (BI) on the *groEL* gene (alignment of 530 bp and HKY+G4 as an evolutionary model) (Figure 3), *sodB* gene (alignment of 592 bp and TPM2+G4 as an evolutionary model) (Figure 4), and 23S–5S intergenic region (alignment of 401 bp and HKY+G4 as an evolutionary model) (Figure 5) grouped the sequences detected in the present study in a new clade sharing the same origin with *E. ruminantium*, with posterior probabilities ranging from 78 to 98%. The genetic divergence between *Ehrlichia* genotypes detected in coatis from the present study and *E. ruminantium* ranged from 0.02 to 0.16 for *groEL, sodB*, and 23S-5S molecular markers (Supplementary Materials).

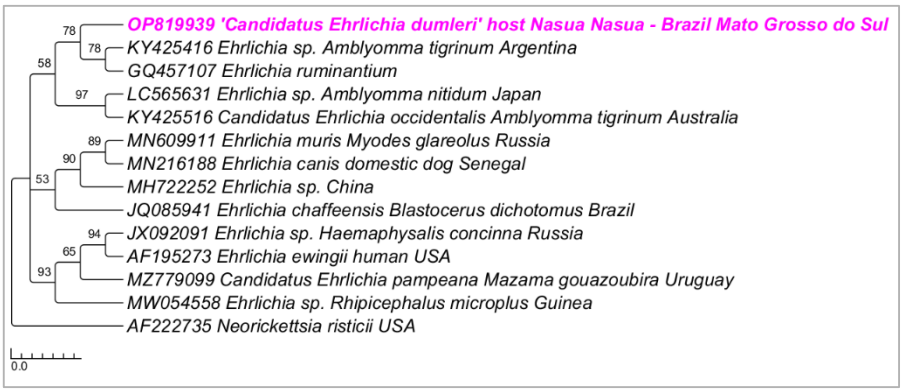

Figure 3. Phylogenetic tree inferred by the Bayesian method and based on the *groEL* gene from *Ehrlichia* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil. Sequences detected in the present study are highlighted in bold pink (VBA).

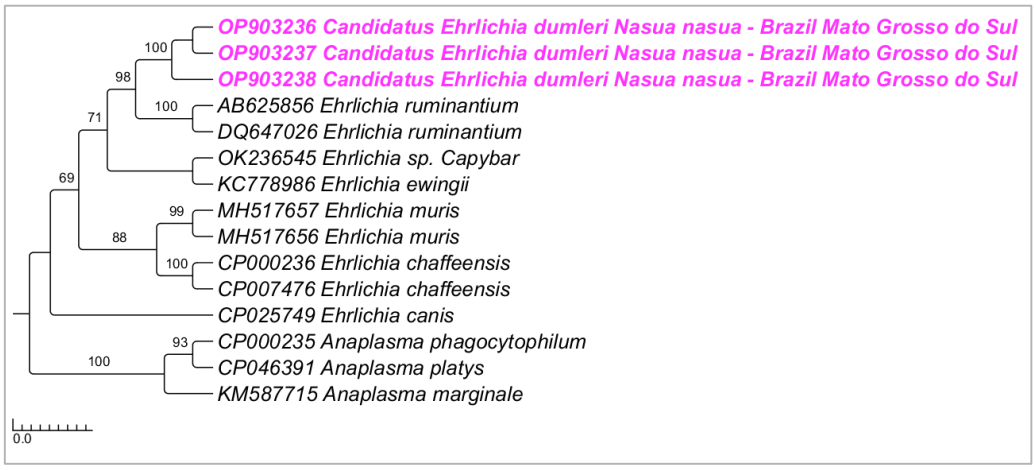

Figure 4. Phylogenetic tree inferred by the Bayesian method and based on the *sodB* gene from *Ehrlichia* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil. Sequences detected in the present study are highlighted in bold pink (VBA).

Figure 5. Phylogenetic tree inferred by the Bayesian method and based on the 23S–5S intergenic region from *Ehrlichia* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil. Sequences detected in the present study are highlighted in bold pink (VBA) and blue (PEP).

Phylogenetic analysis inferred by the Bayesian method (BI) and TN+G4 evolutionary model and based on the *gltA* gene (alignment of 650 bp) showed similar topology to 16S rRNA-based phylogenetic inference, positioning sequences from the present study in a new large clade (92% posterior probability) divided into two sub-clades (Figure 6). The genetic divergence between sequences from these two sub-clades ranged from 0.01 to 0.16 (Supplementary Materials).

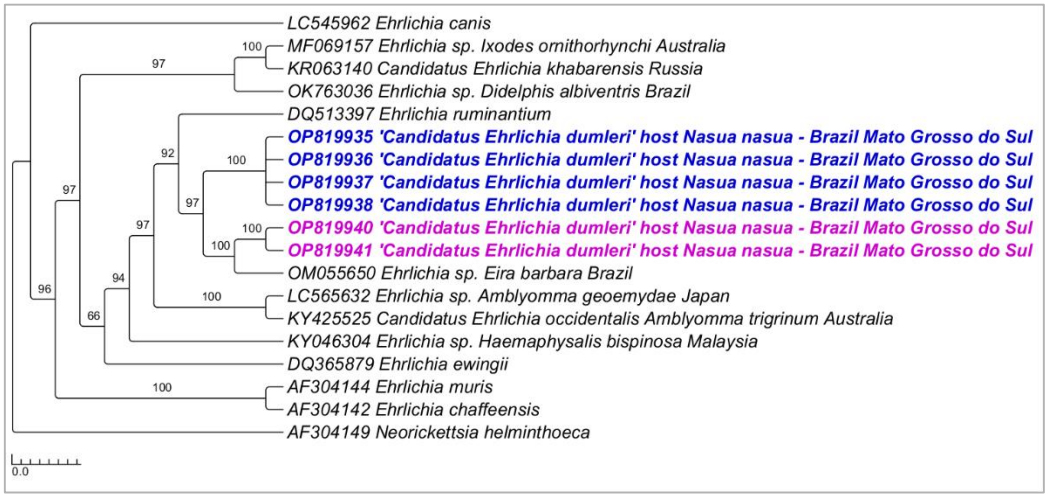

Figure 6. Phylogenetic tree inferred by the Bayesian method and based on the *gltA* gene from *Ehrlichia* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*) sampled in Campo Grande city, Mato Grosso do Sul state, Brazil. Sequences detected in the present study are highlighted in bold pink (VBA) and blue (PEP).

In order to evaluate the genetic distance between the two *Ehrlichia* sp. sub-clades detected in the present study (PEP x VBA), median joining network (MJN) and haplotype analyses with fourteen 16S rRNA sequences that clustered together or in a clade near those sequences detected in coatis (Table 3, Figure 7) was performed. As a result, seven genotypes were found with 34 variable sites. In the MJN analysis, sequences detected in coatis from VBA and the one detected in *E. barbara*, Mato Grosso, Brazil (MZ130191) were represented by the same haplotype (haplotype #4). On the other hand, sequences detected in PEP clustered in two different genotypes (haplotype #1 and #2), also apart from haplotype 3, which was represented by '*Ca. Ehrlichia occidentalis*' (KY425523). Sequences positioned in a clade near sequences detected in coatis (*E. ruminantium* DQ647615, DQ647616, and *Ehrlichia* sp. DQ324367) in the BI were represented by different genotypes (#5, #6, and #7) (Figure 7). The genetic distance (p-distance) between the two detected haplotypes detected from coatis (VBA xPEP) ranged from 0.010 to 0.012 (Supplementary Materials).

Table 3. Polymorphisms and diversity of 16Sr RNA sequences of *Ehrlichia* spp. obtained from coatis (*Nasua nasua*) sampled in the city of Campo Grande, Mato Grosso do Sul state, Brazil.

Gene	bp	N	VS	GC%	H	dh (Mean ± SD)	π (Mean ± SD)	K
16S rRNA	1066	14	34	49.4	7	0.802 ± 0.094	0.001168 ± 0.00170	12.37363

N = number of sequences analyzed, VS = number of variable sites, GC% = C+G content, h = number of haplotypes, dh = diversity of haplotypes, SD = standard deviation, π = nucleotide diversity (per site), K = nucleotide difference number.

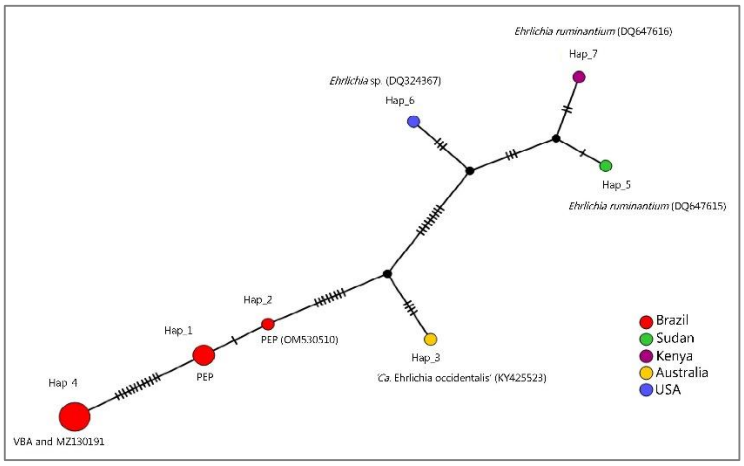

Figure 7. Median joining network containing *Ehrlichia* 16S rRNA sequences representatives of the two clades where sequences detected in coatis were positioned in the Bayesian Inference. While the lines between haplotypes represent mutational steps, the black circles indicate median vectors.

3.2. PCR Assays for Anaplasma sp. Screening and Characterization

Out of 165 coati blood samples, six (3.6%) were positive in the screening nPCR for the 16S rRNA gene of *Anaplasma* spp. [1/63 samples—1.6% from PEP area and 5/102 samples—4.9% from VBA (obtained from five different coatis). No recaptured animal showed positivity in more than one sampling time. Co-positivity for *Ehrlichia* spp. and *Anaplasma* spp. was not found in the sampled animals. Four samples were properly sequenced, and Blast analyses showed 100% of query coverage, 0.0 E-value, and 100% of identity with *Anaplasma* spp. sequences previously detected in ticks and wild animals from Brazil (KY499191; KY391804; KY499201; KY499183). Only one sample amplified for the 23S–5S ITS and Blast results showed 100% of query coverage, 0.0 E-value, and 100% of identity with '*Candidatus* Anaplasma brasiliensis'. All six samples failed to amplify the *groEL* gene fragment.

Phylogenetic analysis inferred by the Bayesian method (BI) and TPM2+I+G evolutionary model and based on the 16S rRNA gene (alignment of 698 bp) grouped the sequences detected in the present study in a large clade (100% posterior probability) with other sequences of *Anaplasma* sp. detected in ticks and wild animals from Brazil (KY499191; KY391804; KY499201; KY499183) (Figure 8). Phylogenetic analysis inferred by the

Bayesian method (BI) and evolutionary model K3P+G4 and based on the 23S–5S ITS region (alignment of 384 bp) grouped the sequences detected in the present study in a large clade (70% posterior probability) with '*Candidatus* Anaplasma brasiliensis' (Figure 9).

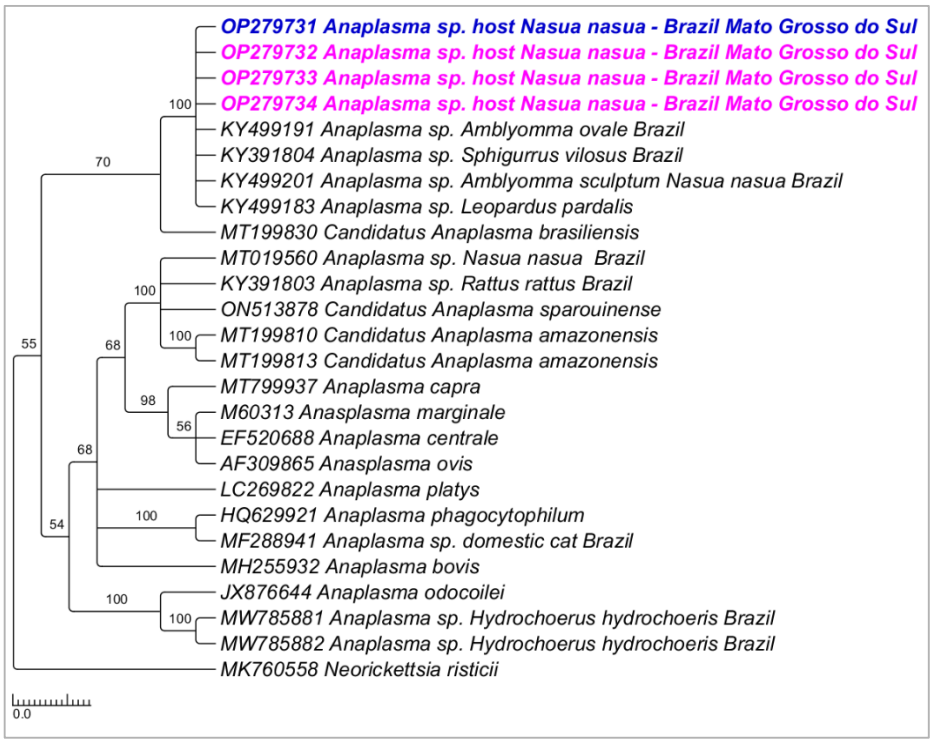

Figure 8. Phylogenetic tree inferred by the Bayesian method and based on the 16S rRNA gene from *Anaplasma* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*). Sequences detected in the present study are highlighted in bold pink (VBA) and blue (PEP).

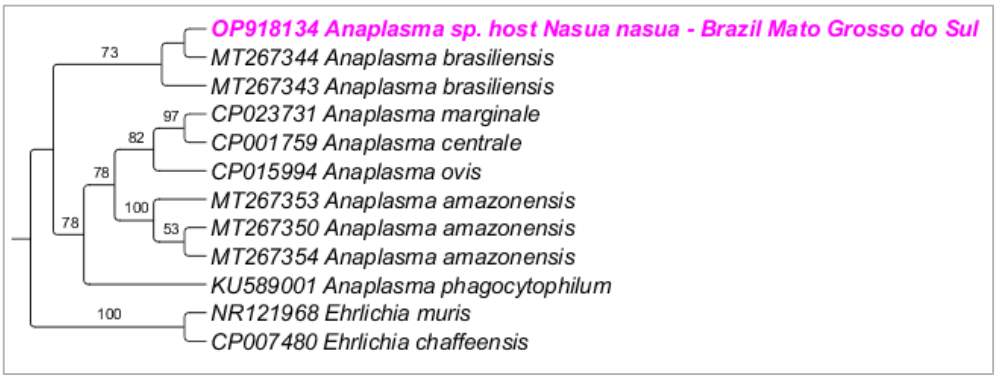

Figure 9. Phylogenetic tree inferred by the Bayesian method and based on the 23S–5S ITS region from *Anaplasma* spp. sequences obtained from blood from ring-tailed coatis (*Nasua nasua*). Sequences detected in the present study are highlighted in bold pink (VBA) and blue (PEP).

4. Discussion

Previous studies conducted in Brazil indicated the occurrence of putative new genotypes of *Ehrlichia* spp. and *Anaplasma* spp. in different orders of wild animals [1,33–42]. These

epidemiological studies indicate an abundant diversity of Anaplasmataceae agents circulating in wild mammals, pointing out the occurrence of putative new species. Regarding this aspect, constant surveillance of Anaplasmataceae agents in biological samples from wild animals shows great importance.

Herein, we were able to detect *Ehrlichia* DNA in 25 (15.1%) blood samples from coatis using a *dsb*-based PCR screening. Such findings represent a higher percentage of positivity when compared with two previous studies performed in Brazil. In the Brazilian Pantanal, a previous study reported the occurrence of antibodies to *E. canis* in one coati (1/31, 3.2%) (titer of 640) as well as the molecular detection of a *Ehrlichia* 16S rDNA genotype phylogenetically related to multiple *Ehrlichia* spp. previously detected in ticks and wild rodents from Brazil [39]. Moreover, *Ehrlichia/Anaplasma* 16S rDNA was detected in 1/18 coati blood sampled in Iguazu National Park, southern Brazil, albeit without sequencing [43]. In the present study, we were able to sample a higher number of animals when compared with the two above-mentioned studies from Brazil (165 blood samples analyzed herein, in contrast with 31 blood samples performed by Sousa et al. [39] and 18 by Collere et al. [43]). This higher number of sampling procedures might explain the higher percentage of positive animals found in the present study. The two previous studies and the present one were performed in different biomes: while the study conducted by Sousa et al. [39] took place in Pantanal, the latter performed by Collere et al. [43] was carried out in the Atlantic forest. The present study was conducted in the Cerrado biome, an area characterized by grassland to a nearly closed canopy of medium-height trees overlying grass with a tropical savanna climate [4]. Although these three studies were performed with the same animal species, it is known that tick diversity changes according to the studied biome [3,8,39,44,45]. Animals from the present study were found parasitized by non-identified *Amblyomma* sp. larvae, *Amblyomma dubitatum*, and *Amblyomma sculptum* nymphs, and *A. sculptum* and *Amblyomma ovale* adults [8]. Since the vector of the detected *Ehrlichia* sp. is not known yet, the studied biome and, consequently, tick diversity, together with the higher number of sampled animals, might have influenced the higher percentage of positive animals in the Cerrado biome. It is also worth mentioning that the applied technique might have influenced the percentage of positive animals. For instance, nested PCR is highly sensitive, and in the case of *E. canis* detection, it is capable of detecting 0.2 pg of purified DNA, while a conventional PCR can only detect values above 20 pg [46]. Quantitative PCR (qPCR) can also be used to increase the sensibility of the diagnostic tests and screening, and in some cases, showed a higher sensitivity compared to previously described gel-based PCR, RNA, PCR-ELISA, and hybridization assays [47]. However, the ability of previously standardized qPCR protocols to

catch novel Anaplasmataceae agents should be further investigated. In a previous study, a multiplex qPCR based on the *groEL* gene and validated for the main *Ehrlichia* and *Anaplasma* species of medical and veterinary importance [37] was unable to catch putative novel *Ehrlichia* and *Anaplasma* genotypes described in mammals from the Superorder Xenarthra in Brazil [41].

We observed a different prevalence of positive animals according to the sampling spot [4/63 samples—6.3% from the PEP area (from four different coatis) and 21/102 samples—20.6% from VBA (obtained from 16 different coatis). Although sampling areas are in Campo Grande city, Cerrado biome, they present very different characteristics. PEP is a preserved forest area, being an important refuge for wildlife, where many species of mammals can be found, such as ring-tailed coatis, anteaters, capybaras, opossums, bats, and also many species of birds and reptiles [4,48–51]. Contrary, VBA is a more anthropized area (residential area), surrounded by three forest fragments and one area used for military training. There is a residential complex inhabited by many families and domestic animals. The houses are not fenced, and they all have a trash can at the front. Coatis have access to the outside of the houses, and they were observed during field activities walking around the houses and also searching for food in the dumpsters [8]. This difference in the prevalence between the two areas may be explained by a phenomenon called ‘dilution effect’ [52–54]. Preserved areas (e.g., PEP) are characterized by high species richness of different hosts for ectoparasites and pathogens or are likely to contain a high proportion of hosts that are inefficient in transmitting the disease agent to a feeding vector. With a higher abundance of weakly competent reservoir species, the stronger the dilution effect is, which may decrease the probability of disease transmission [54].

Interestingly, the present study shows, for the first time, transient positivity for *Ehrlichia* sp. in coati blood samples. Three animals sampled at VBA were PCR-positive at different times of sampling. All three animals were positive in March 2019 and two animals were in April 2019. Due to the small number of recaptured/positive animals, and since the captures were performed by chance on different days, it is not possible to infer any type of correlation according to the sampling period and PCR positivity. Besides that, the results found herein are influenced by the limit of the detection of the PCR protocols used. Nevertheless, these data indicate that *N. nasua* may play a role in the maintenance of this new Anaplasmataceae agent in the wild environment.

Phylogenetic analyses positioned *Ehrlichia* sp. detected from coatis in the present study in a new clade, originated as a sister clade to *E. ruminantium*. It is noteworthy that all phylogenetic inferences based on different molecular markers showed the same topology,

with the establishment of a new distinct clade. Interestingly, two branches were observed on the phylogenetic inferences based on 16S rRNA and *gltA* genes and 23S-5S intergenic region, according to the sampling spot (PEP and VBA). These findings may indicate the occurrence of distinct genotypes of a unique putative novel Anaplasmataceae agent, which is proposed here as '*Candidatus* Ehrlichia dumleri', in honor of Dr. John Stephen Dumler and his great contribution to the knowledge of this group of proteobacteria. Indeed, while the genetic divergence between these two genotypes ranged from 0.010 to 0.013, values ranging from 1.2 to 1.3% were found when comparing the newly described genotypes and '*Candidatus* Ehrlichia occidentalis'. Moreover, the haplotype analyses based on the 16S rRNA sequences corroborate the presence of two distinct genotypes, also according to the sampling spot.

Recently, several '*Candidatus* Ehrlichia' have been proposed and detected in biological samples from wild animals by using different molecular target genes. For instance, '*Candidatus* Ehrlichia regneryi' was detected in spleen samples of dromedary camels (*Camelus dromedarius*) in Saudi Arabia using two genetic markers (16SrRNA and *groEL*) [55]. '*Candidatus* Ehrlichia pampeana' was detected in *Haemaphysalis juxtakochi* and in gray Brocket Deer (*Mazama gouazoubira*) from Uruguay using three loci (16S rRNA, *dsb,* and *groEL*) [56]. '*Candidatus* Ehrlichia hydrochoerus' was detected in blood samples from capybaras (*Hydrochoerus hydrochaeris*) from Brazil, using four loci (16S rRNA, *dsb, groEL* and *sodB*) [57]. In the present study, we were able to perform the molecular characterization using six different molecular targets, including a large fragment of 16S rRNA, *dsb*, s*odB*, *groEL*, *gltA* genes and the 23S-5S intergenic space region (ITS). Phylogenetic analyses based on all six molecular markers showed the same topology, supporting the description of the new '*Candidatus* Ehrlichia dumleri' presented herein.

The present work describes a low percentage of positive animals for *Anaplasma* spp. (6/165—3.6%) by using a screening nPCR based on the 16S rRNA gene. Sousa et al. [39] detected *Anaplasma* spp. in 22% (7/31) coatis sampled in the Brazilian Pantanal, using an nPCR also based on the 16S rRNA gene. Phylogenetic inferences grouped the sequences detected in coatis from Pantanal in two different clades: one clade related to *A. phagocytophilum* and another one clustering with *Anaplasma* sp. previously detected in different wild animal species, including rodents, marsupials, and carnivores [39]. The present work showed the detection of an *Anaplasma* sp. closely related to '*Candidatus* Anaplasma brasiliensis', which was previously detected in anteaters in the state of São Paulo [41], and *Anaplasma* sp. detected in other wild animals from Brazil. Recently, an unusual case of human anaplasmosis caused by '*Candidatus* Anaplasma sparouinense', which was

genetically related to '*Candidatus* Anaplasma amazonensis' was recently described in sloths from the Brazilian Amazon [41], was reported in the Amazon of French Guiana [2]. Future studies should be performed in order to check if wild animals may represent a source of infection by these novel neotropical *Anaplasma* to humans.

5. Conclusions

Anaplasmataceae agents were detected in blood samples from free-ranging coatis sampled in Midwestern Brazil. A higher positivity for *Ehrlichia* spp. was found in the sampled coatis when compared to that one found for *Anaplasma* spp. Phylogenetic analyses based on six molecular markers (16S rRNA, *dsb*, s*odB*, *groEL*, *gltA,* and the 23S-5S ITS) positioned the novel *Ehrlichia* sp. detected in the sampled coatis into a new clade and supported the description of '*Candidatus* Ehrlichia dumleri', a putative novel Anaplasmataceae species that appeared as a sister clade to *E. ruminantium*. Phylogenetic analyses based on both 16S rRNA gene and 23S–5S intergenic region grouped the *Anaplasma* sp. detected in coatis with the previously described '*Candidatus* Anaplasma brasiliensis'.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Blast results from positive animals for *Ehrlichia* sp., with GenBank accession number from the present study, localization, gene, fragment size, organism, % of query coverage, E-value, % of identity and GenBank accession number of the pathogen; Table S2: Pairwise genetic distances matrix among *Ehrlichia* sp. that clustered close to sequences detected at the present study at based on *dsb* gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold pink (Air Force Private Area); Table S3: Pairwise genetic distances matrix among *Ehrlichia* sp. that clustered close to sequences detected at the present study at based on large fragment of 16SrRNA gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold blue (*Parque Estadual do Prosa*) and pink (Air Force Private Area); Table S4: Pairwise genetic distances matrix among *Ehrlichia* sp. sequence that clustered close to sequence detected at the present study at based on *groEL* gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequence detected at the present study is highlighted in bold pink (Air Force Private Area); Table S5: Pairwise genetic distances matrix among *Ehrlichia* sp. sequences that clustered close to sequences detected at the present study at based on *sodB* gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold pink (Air Force Private Area); Table S6: Pairwise genetic distances matrix among *Ehrlichia* sp. sequences that clustered close to sequences detected at the present study at based on 23S-5S intergenic region. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold pink (Air Force Private Area); Table S7: Pairwise genetic distances matrix among *Ehrlichia* sp. sequences that clustered close to sequences detected at the present study at based on *gltA* gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold blue (*Parque Estadual do Prosa*) and pink (Air Force Private Area).

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project administration: M.R.A., H.M.H., and R.Z.M.; supervision: M.R.A., H.M.H., and R.Z.M.;writing—original draft: M.R.A. and L.P.; writing—review and editing: M.R.A., R.Z.M., H.M.H., L.P., A.C.C., W.T.G.B., and G.C.d.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All obtained sequences were submitted to GenBank with following accession numbers: *Ehrlichia* sp. *dsb* (OP819944–OP819946), *Ehrlichia* sp. 16S rRNA (OM530509-OM530517), *Ehrlichia* sp. *groEL* (OP819939), *Ehrlichia* sp. *sodB* (OP903236-OP903238), *Ehrlichia* sp. 23S-5S (OM717254-OM717255), *Ehrlichia* sp. *gltA* (OP819935-OP919938, OP819940-OP819941), *Anaplasma* sp. 16S rRNA (OP279731-OP279734), and *Anaplasma* sp. 23S-5S (OP918134) and could be accessed through https://www.ncbi.nlm.nih.gov/genbank/.

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Supplementary Material

Multi-Locus Sequencing Reveals Putative Novel Anaplasmataceae Agents, '*Candidatus* Ehrlichia dumleri' and *Anaplasma* sp., in Ring-Tailed Coatis (Carnivora: *Nasua nasua*) from Urban Forested Fragments at Midwestern Brazil

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Table S1. Blast results from positive animals for *Ehrlichia* sp., with GenBank accession number from the present study, localization, gene, fragment size, organism, % of query coverage, E-value, % of identity and GenBank accession number of the pathogen.

Sample ID	Local	Gene	Fragment size (bp)	Scientific name	% Query coverage	Evalue	% Identity	GenBank accession number
OP819944	VBA*	*dsb*	394	*Ehrlichia* sp. sloth Brazil	70%	1e-101	94%	MH236883
OP819945			363			3e-87	94.09%	
OP819946			395			2e-100	93.60%	
OM530509	PEP**	16SrRNA	1152	*Ehrlichia* sp. *Eira barbara* Brazil	99%	0.0	98.26%	MZ130191
OM530510			1137		100%		98.50%	
OM530511			1191		99%		98.65%	
OM530512			1136		100%		98.98%	
OM530513	VBA		1080		100%	0.0	100%	
OM530514			1131					
OM530515			1187					
OM530516			1139					
OM530517			1133					
OP819939	VBA	*groEL*	530	*Ehrlichia* sp.	100%	0.0	92.26%	KY425416
	VBA	*sodB*	589	*Ehrlichia ruminantium*	99%	8e-175	85.94%	AB625856
			579				85.66%	DQ647026
			576				85.74%	
OM717254	PEP	ITS-23S5S	387	*Ehrlichia ruminantium*	100%	0.0	97.18%	CP063045
OM717255	VBA		363				97.27%	CR925677
OP819935	PEP	*gltA*	583	*Ehrlichia ruminantium*	100%	1e-147	90%	DQ513397
OP819936			566				90%	
OP819937			631				90%	
OP819938			524				90%	
OP819940	VBA		485	*Ehrlichia* sp.				

OP819941		637	*Eira barbara* Brazil	97%	0.0	99.26%	OM055650

*Vila da Base Aérea; **Parque Estadual do Prosa

Table S2. Pairwise genetic distances matrix among *Ehrlichia* sp. that clustered close to sequences detected at the present study at based on *dsb* gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold pink (Air Force Private Area).

	OP819944	OP819945	OP819946	MH236883	OM863958	MH236882
OP819945	0,00					
OP819946	0,00	0,00				
MH236883	0,05	0,06	0,05			
OM863958	0,15	0,16	0,15	0,14		
MH236882	0,17	0,17	0,17	0,15	0,03	
KY413807	0,18	0,18	0,18	0,14	0,08	0,10

Table S3. Pairwise genetic distances matrix among *Ehrlichia* sp. that clustered close to sequences detected at the present study at based on large fragment of 16SrRNA gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold blue (*Parque Estadual do Prosa*) and pink (Air Force Private Area).

	OM530509	OM530510	OM530511	OM530512	OM530513	OM530514	OM530515	OM530516	OM530517	MZ130191
OM530510	0,000									
OM530511	0,000	0,001								
OM530512	0,000	0,001	0,000							
OM530513	0,012	0,013	0,012	0,013						
OM530514	0,012	0,012	0,012	0,012	0,000					
OM530515	0,012	0,012	0,012	0,012	0,000	0,000				
OM530516	0,011	0,012	0,011	0,012	0,000	0,000	0,000			
OM530517	0,011	0,012	0,011	0,012	0,000	0,000	0,000	0,000		

MZ130191	0,013	0,014	0,013	0,014	0,000	0,000	0,002	0,002	0,000	
KY425523	0,013	0,012	0,013	0,013	0,017	0,017	0,016	0,017	0,017	0,018

Table S5. Pairwise genetic distances matrix among *Ehrlichia* sp. sequences that clustered close to sequences detected at the present study at based on *sodB* gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold pink (Air Force Private Area).

	OP903236	OP903237	OP903238	AB625856
OP903237	0,00			
OP903238	0,00	0,00		
AB625856	0,16	0,16	0,16	
DQ647026	0,15	0,15	0,15	0,00

Table S6. Pairwise genetic distances matrix among *Ehrlichia* sp. sequences that clustered close to sequences detected at the present study at based on 23S-5S intergenic region. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold pink (Air Force Private Area).

	OM717254	OM717255	CP063045
OM717255	0,00		
CP063045	0,02	0,02	
CR925677	0,02	0,02	0,00

Table S7. Pairwise genetic distances matrix among *Ehrlichia* sp. sequences that clustered close to sequences detected at the present study at based on *gltA* gene. Pairwise genetic distances were obtained using the p-distance method in MEGA X. Sequences detected at the present study are highlighted in bold blue (*Parque Estadual do Prosa*) and pink (Air Force Private Area).

	OP819935	OP819936	OP819937	OP819938	OP819940	OP819941	OM055650
OP819936	0,00						
OP819937	0,00	0,00					
OP819938	0,00	0,00	0,00				
OP819940	0,14	0,16	0,15	0,14			
OP819941	0,15	0,16	0,15	0,15	0,00		

OM055650	0,13	0,15	0,14	0,13	0,01	0,01	

Capítulo 6* - *Neorickettsia* sp. in coatis (*Nasua nasua*) from Brazil

Neorickettsia sp. em quatis (*Nasua nasua*) do Brasil

* Esse capítulo corresponde ao manuscrito submetido à Revista Brasileira de Parasitologia Veterinária

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Abstract

The genus *Neorickettsia* comprises trematode-associated bacteria that can cause diseases in animals and humans. Despite the detection of *Neorickettsia* antigens by immunohistochemistry in the intestine of ring-tailed coatis maintained in captivity in southern Brazil, the molecular identity of the bacteria in procyonids from South America remains elusive. The present work aimed to investigate the occurrence of *Neorickettsia* sp. in coatis' blood samples from Midwestern Brazil. Between March 2018 and January 2019, animals were captured and re-captured in two Cerrado areas (*Parque Estadual do Prosa* PEP and *Vila da Base Aérea* VBA) located in Campo Grande city, Mato Grosso do Sul State, Midwestern Brazil. All captures and recaptures were performed by convenience, and recapture times were performed by chance. DNA from 165 blood samples were subjected to a nested(n) PCR targeting a fragment of the 16S rRNA gene of *Neorickettsia* sp. The positives samples for the 16S rRNA were further subjected to PCR assays targeting the nearly full-length 16S rRNA, *p51kDa* and *groEL* genes. Six (3.6% - 5 from VBA and 1 from PEP) samples from different coatis were positive in the nPCR based on the 16S rRNA. The obtained sequences (~500 bp) showed >99% identity with *N. risticii*. This is the first molecular detection of *Neorickettsia* sp. in coatis from Brazil. The real identity of the bacteria detected in ring-tailed coatis should be further investigated through isolation and WGS.

Keywords: Anaplasmataceae, Procyonidae, Midwestern Brazil

Resumo

O gênero *Neorickettsia* compreende bactérias associadas a trematódeos que podem causar doenças em animais e humanos. Apesar da detecção de antígenos de *Neorickettsia* por imuno-histoquímica no intestino de quatis mantidos em cativeiro no sul do Brasil, a identidade molecular da bactéria em procionídeos da América do Sul permanece indefinido. O presente trabalho teve como objetivo investigar a ocorrência de *Neorickettsia* sp. em amostras de sangue de quatis do Centro-Oeste do Brasil. Entre março de 2018 e janeiro de 2019, os animais foram capturados e recapturados em duas áreas de Cerrado (Parque Estadual do Prosa PEP e Vila da Base Aérea VBA) localizadas na cidade de Campo Grande, Mato Grosso do Sul, Centro-Oeste do Brasil. Todas as capturas e recapturas foram realizadas por conveniência e os tempos de recaptura foram realizados ao acaso. O DNA de 165 amostras de sangue foi submetido a nested (n) PCR visando um fragmento do gene 16S rRNA de *Neorickettsia* sp. As amostras positivas para o rRNA 16S foram posteriormente submetidas a ensaios de PCR visando um fragmento longo do gene 16S rRNA e os genes *p51kDa* e *groEL*. Seis (3,6% - 5 de VBA e 1 de PEP) amostras de quatis diferentes foram positivas na nPCR baseada no rRNA 16S. As sequências obtidas (~500 pb) apresentaram >99% de identidade com *N. risticii*. Esta é a primeira detecção molecular de *Neorickettsia* sp. em quatis do Brasil. A real identidade da bactéria detectada em quatis deve ser melhor investigada por meio de isolamento e WGS.

Palavras-Chave: Anaplasmataceae, Procionídeo, Centro-oeste brasileiro

Apart from the other Anaplasmataceae agents (Order Rickettsiales), which are transmitted by blood sucking arthropods, the genus *Neorickettsia* comprises trematode-associated bacteria that can cause diseases in animals and humans (Dumler et al., 2001). Although several molecular studies reported the presence of *Neorickettsia* DNA in different hosts, due to the great difficulty in cultivating and sequencing the complete genome of these bacteria, only four species have been fully characterized, namely *Neorickettsia sennetsu,* that causes the Sennetsu neorickettsiosis in humans (Dittrich et al., 2015), *Neorickettsia helminthoeca* causing Salmon Poisoning Disease (SPD) in dogs Headley et al., 2011, and *Neorickettsia risticii* and *Neorickettsia findlayensis* that are the etiological agents of Potomac horse fever (PHF) (Teymournejad et al., 2020).

In Brazil, *N. risticii* has already been molecularly detected in horses presenting clinical signs resembling Potomac fever in the states of Rio Grande do Sul (Coimbra et al., 2006) and Rio de Janeiro (Paulino et al., 2020). *Neorickettsia risticii* DNA was also detected in *Heleobia piscium*, *Heleobia parchappei* and *Heleobia davisi* snails in the state of Rio Grande do Sul, where an outbreak of PHF had been previously reported (Coimbra et al., 2006). Cercariae of trematodes of the species

Parapleurolophocercous cercariae identified in the snail *H. piscium* also contained *N. risticii* DNA (Coimbra et al., 2006). Recently, *Neorickettsia* sp. DNA was detected in 57 (13.63%) out of 418 biological samples from fruit bats sampled in a periurban area of Campo Grande city, Mato Grosso do Sul state, midwestern Brazil (Ikeda et al., 2021).

SPD caused by *N. helminthoeca* is referred as an endemic disease in dogs in Western USA and Canada (Headley et al., 2011). In Brazil, Headley et al. (2006) found anatomopathological lesions in 10 dogs from the city of Maringá, Paraná state, which were consistent with alterations associated with SPD. Based on PCR assays targeting *rpoB* and *groEL* genes, the presence of *N. helminthoeca* DNA was detected in dog tissues with pathologic lesions consistent with SPD (Headley et al., 2006). *Neorickettsia helminthoeca* antigens were detected by immunohistochemistry in the intestine of three ring-tailed coatis kept in a conservationist breeding facility in Foz do Iguaçu, southern Brazil. Intestine and spleen fragments were positive in a PCR for *N. helminthoeca* based on the 16S rRNA gene, but due to the low quality of the amplicons obtained, no sequence was obtained (Headley et al., 2018). The present work aimed to investigate the occurrence of *Neorickettsia* sp. in coatis' blood samples from Midwestern Brazil.

Between March 2018 and January 2019, coatis were sampled every three months during 10 consecutive days in two urban areas: a conservation unit, namely 'Parque Estadual do Prosa' (PEP) (-20.44987, -54.56529), and a residential area, namely 'Vila da Base Aérea' (VBA) (-20.47163, -54.65405), located in Campo Grande city, state of Mato Grosso do Sul State, Midwestern Brazil. The animals were anesthetized with an association of Tiletamine hydrochloride and Zolazepam hydrochloride (Telazol, Zoetis® - 7 mg/Kg, Intramuscularly). For identification in future recaptures, coatis were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue. Age was estimated according to Olifiers et al. (2010). In total, 97 different coatis were sampled (42 PEP and 55 VBA; 56 females and 41 males; and 70 adults and 27 subadults). Coatis were recaptured by chance, totaling 163 blood samples in total (first capture and recaptures). Blood was sampled from femoral vein using tubes containing EDTA (Ethylenediamine Tetraacetic Acid) (Perles et al., 2022).

DNA was extracted from 165 blood samples using Illustra Blood Mini Kit (GE Healthcare®, Chicago, IL, USA), according to the manufacturer's instructions. All DNA samples were initially submitted to a PCR targeting the mammal endogenous *gapdh* (Glyceraldehyde 3-phosphate dehydrogenase) gene (Birkenheuer et al., 2003). Positive samples were subjected to a nested (n) PCR targeting a fragment (~ 500 bp) of the 16S rRNA gene of *Neorickettsia* sp. (Chae et al., 2003). The positives samples for the 16S rRNA were further subjected to PCR assays targeting the nearly full-length 16S rRNA (Kanter et al., 2000), *p51kDa* and *groEL* genes (Barlough et al., 1998; Gibson et al., 2011; Cicuttin et al., 2013).

All 165 coati blood DNA samples were positive in the PCR targeting the *gapdh* gene. Six (3.6% - 5 from VBA and 1 from PEP) different coatis were positive in the nPCR based on the 16S rRNA, being 5 positive at the first capture, and one positive only on the second capture. The obtained sequences (~500 bp) showed >99% (query coverage of 100% and E-value of 0.0) identity with *N. risticii*. Unfortunately, all six samples were negative in the additional PCR protocols targeting the *p51kDa*, *groEL*, and near-full 16S rRNA genes, precluding robust phylogenetic inferences. The sequences generated and analyzed during the current study are available in the NCBI GenBank Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession numbers: OP980542, OP980543, OP980544, OP980545, OP980546 and OP980547.

Despite the sequences obtained herein showed high identity with *N. risticii*, Dumler et al. 2001, demonstrated phylogenetic heterogeneity among *N. risticii* 16S rRNA sequences, showing that mutations of up to 15 nucleotides can be present, with the formation of different clades within the same species. In fact, a new *Neorickettsia* species has been recently described in an area where *N. risticii* was incriminated as the only etiological agent of PHF in horses. Based on phylogenetic inferences on nearly complete 16S rRNA, *p51*, *Ssa3* and *Ssa1* genes, isolation and Whole Genome Sequencing (WGS), Teymournejad et al. 2020 described *Neorickettsia findlayensis* in horses from Canada. Although the positive samples from the present study showed >99% of identity with *N. risticii* available at GenBank, the real identity of the bacteria detected in ring-tailed coatis should be further investigated through isolation and WGS.

Neorickettsia sp. was molecularly detected in wild ring-tailed coatis blood samples from a peri-urban area in midwestern Brazil. Since *Neorickettsia* sp. was molecularly detected in bats in the same studied area by Ikeda at al. (2021) and in the present work in wild coatis, we predict that this Anaplasmataceae agents might be maintained in peri-urban areas from Midwestern Brazil by an intricate transmission cycle involving free-living mammals. Future studies should be performed in order to isolate the detected agent in order to shed some light on the real identity of *Neorickettsia* circulating in wild animals from Brazil.

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Ethics declaration

All methods were carried out in accordance with relevant guidelines and regulations and were approved by the “Instituto Chico Mendes de Biodiversidade” (ICMBio) (SISBIO 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB 001/2018) and ‘Sistema Nacional de Gestão de Patrimônio Genético’ (ABDF0B5).

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship or publication of this article.

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Capítulo 7* - Molecular survey of hemotropic *Mycoplasma* spp. and *Bartonella* spp. in coatis (*Nasua nasua*) from central-western Brazil

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Abstract: Even though previous works showed molecular evidence of hemotropic *Mycoplasma* spp. (hemoplasmas) in ring-tailed coatis (*Nasua nasua*) from Brazil, *Bartonella* sp. has not been reported in these mammals so far. The present study aimed to detect the above mentioned agents in coatis' blood and associated ectoparasites, assessing the association between these infections and red blood parameters. Between March 2018 and January 2019, coati (n=97) blood samples, *Amblyomma* sp. ticks (2,242 individual ticks, resulting in 265 pools) and *Neotrichodectes pallidus* louse (n= 59) were collected in forested urban areas from midwestern Brazil. DNA extracted from coatis' blood and ectoparasite samples were submitted to quantitative PCR (qPCR) (16S rRNA) and conventional PCR (cPCR) (16S rRNA and 23S rRNA) for hemoplasmas and qPCR (*nuoG* gene) and culturing (only blood) for *Bartonella* spp.. Two different hemoplasma genotypes were detected in blood samples: 71% coatis positive for myc1 and 17% positive for myc2. While 10% ticks were positive for hemoplasmas (myc1), no louse was positive. The estimate bacterial load of hemoplasmas showed no association with anemia indicators. All coatis were negative for *Bartonella* sp. in qPCR assay and culturing, albeit two *Amblyomma* sp. larvae pools and 2 *A. dubitatum* nymph pools were positive in the qPCR. The present work showed high occurrence of hemoplasmas, with two distinct hemoplasma genotypes, in coatis from forested urban areas from midwestern Brazil.

Key-Words: Hemotropic *Mycoplasma* spp., *Amblyomma* spp., bartonellae, *Neotrichodectes pallidus*, Midwestern Brazil.

1. Introduction

Hemotropic mycoplasmas (hemoplasmas) (Mycoplasmatales: Mycoplasmataceae) are obligate epi-erythrocytic bacteria that might be transmitted by hematophagous arthropods or agressive interactions between animals [1, 2]. Previous works showed a high occurrence of hemoplasmas among raccoons (*Procyon lotor*) in the USA, with description of five novel 16S rRNA hemoplasmas genotypes [3]. Similarly, Sousa et al. [4] reported a high occurrence of the bacteria in ring-tailed coatis (Procyonidae: *Nasua nasua*; hereafter "coati") in the Brazilian Pantanal, with the description of two 16S rRNA genotypes. In Paraná state, southern Brazil, hemoplasma was detected in captive coatis [5] and a putative novel, namely '*Candidatus* Mycoplasma haematonasua', was evidenced through phylogeny based on two molecular markers (16S rRNA and 23S rRNA) in wild coatis [6].

Infections by hemoplasmas in domestic animals can be manifested by acute and chronic illnesses. Acute infections are characterized by anemia and acute hemolysis, anorexia, lethargy, dehydration, weight loss, and, in some cases, sudden death [7]. Chronic infection, on the other hand, can occur in apparently healthy animals, with manifestation of clinical signs in animals that have undergone a splenectomy procedure or immunossupression. The severity of the disease will depend on the strain or species of hemoplasma involved [7]. In wild animals, hemoplasma pathogenicity is poorly known, but infections appear to be subclinical [2]. Therefore, to understand how the bacteria affects wild animals' health, studies assessing clinical and hematological parameters of infected animals are needed.

The genus *Bartonella* spp. (Rhizobiales: Bartonellaceae) encompasses Gram-negative, facultative intracellular α-proteobacteria that primarily infect erythrocytes and endothelial cells of a wide variety of mammals [8]. With at least 45 species described, they are considered re-emerging zoonotic agents, which can cause a plethora of clinical signs, which varies from self-limiting manifestations to fatal systemic diseases [8,9]. The transmission of *Bartonella* spp. is also associated with hematophagous arthropod vectors, such as fleas, lice, sandflies [10] and, more recently, to ticks [11]. Considering the large number of mammal reservoirs and arthropod vectors, the transmission of bartonellae leads to a challenging epidemiological scenario, especially in regions where wild animals are in close contact with humans and domestic animals. Regarding the occurrence of bartonellae in Procyonidae mammals, *Bartonella henselae* and *Bartonella koehlerae* were detected in *P. lotor* in the state of Colorado and St. Simons Island, USA [12, 13].

To the best of authors' knowledge, there are no reports of *Bartonella* sp. in Procyonidae mammals from Brazil and, although hemoplasmas have been reported in coatis from Brazil, there is no information about the infection on health parameters of naturally infected animals. The present study aimed to: i) estimate the fluctuation of hemoplasmas and *Bartonella* spp. bacterial load in free-living coatis in a longitudinal study in two urban areas on central-western Brazil by quantitative PCR (qPCR); ii) investigate the presence of hemoplasmas and *Bartonella* spp. DNA in ectoparasites collected from coatis; iii) assess the correlation between the two bacterial infections and coatis' red blood cells parameters.

2. Material and Methods

2.1. Blood and ectoparasites sampling

Between March 2018 and January 2019, coatis were sampled every three months during 10 consecutive days in two urban areas: a conservation unit 'Parque Estadual do Prosa' (PEP) (-20.44987, -54.56529) and a residential 'Vila da Base Aérea' (VBA) (-20.47163, -54.65405), both located in Campo Grande city, Mato Grosso do Sul State, Midwestern Brazil. They were anesthetized with an association of Tiletamine hydrochloride and Zolazepam hydrochloride (Telazol, Zoetis® - 7 mg/Kg, Intramuscularly) and were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue between the shoulder blades [14]. Age was estimated according to Olifiers et al. [15] in three groups: adults, subadults and puppies.

In total, 97 different coatis were sampled (42 PEP and 55 VBA; 56 females and 41 males; and 70 adults and 27 subadults). Coatis were recaptured by chance, totalizing 163 blood samples in total (first capture and recaptures). Blood was sampled from femoral vein using tubes containing EDTA (Ethylenediamine Tetraacetic Acid), and then placed into RNAse/DNAse free cryotubes and stored in an -80°C freezer until culturing and molecular analyses. The entire body of the capture animals was inspected, and ectoparasites were collected and stored in 100% ethanol (Merck®) for taxonomic identification [16, 17] and further molecular analyses.

Ticks collected (n= 2,242) were identified as *Amblyomma* spp. larvae (n=838), *Amblyomma sculptum* nymphs (n=1241), *Amblyomma dubitatum* nymphs (n= 150). Thirteen adult ticks were identified as *A. sculptum* (n= 3 males and n= 5 females) and 2 males and 3 females of *Amblyomma ovale* [14]. Lice collected (n= 59) were identified as *Neotrichodectes (Nasuicola) pallidus* (n= 27 nymphs, n= 15 females and n= 17 males) *(manuscript submitted*).

2.2. DNA extraction from coatis' blood and ectoparasite samples, and conventional PCR (cPCR) for mammal gadph*, tick 16S rRNA*, *and insects* cox-1 *endogenous genes*

DNA was extracted from 200 µL of blood using Illustra Blood Mini Kit (GE Healthcare®, USA), according to the manufacturer's instructions. In the case of ticks, while DNA from tick larvae and nymphs was extracted in pools up to 10 and 5 individuals, respectively, collected from the same host, DNA was extracted from adult individually. Regarding lice, specimens collected from individual coatis were subjected to DNA extraction individually; when more than one developmental stage was found in individual coatis, DNA was extracted from louse pools (e.g., pools of nymphs and pools of female adults were extracted separately from an individual). DNA extraction of ticks and lice was performed using Biopur Mini Spin Plus kit (Mobius®, Brazil), according to the manufacturer's instructions. DNA extracted samples were subjected to endogenous gene control cPCR assays {*gapdh* gene (Glyceraldehyde 3-phosphate dehydrogenase) for blood [18], 16S rRNA gene for ticks [19] and *cox1* gene for louse [20]}. Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in all cPCR assays.

2.3. Quantitative PCR assay (qPCR) and molecular characterization of hemoplasmas

In order to detect and quantify the presence of hemoplasmas DNA, positive samples in cPCR assays for the endogenous genes were subjected to a Sybr Green quantitative PCR (qPCR) assay based on the 16S rRNA gene for pan-hemoplasmas [21]. All analyses were performed according to the standards established by MIQE (Minimum Information for Publication of Quantitative real-time PCR Experiments) [22]. Each DNA sample was subjected to qPCR assay in duplicate. The amplification reactions were carried out in a CFX96 Thermal Cycler (BioRad®, USA). Serial dilutions were performed to construct standard curves with different concentrations (2.0×10^7 to 2.0×10^0 copies/µL) of a plasmid encoding a fragment of 104 bp of the 16S rRNA gene of *Mycoplasma haemofelis* (pIDTSMART; Integrated DNA Technologies), diluted in Tris-EDTA (TE; 10 mmol/l, Tris-HCl, 0.1 mmol/l, EDTA) (pH 8.0). Ultra-pure sterile water was used as a negative control.

Due to financial limitations, it was not possible to sequence all positive samples. Therefore, 15 samples were randomly selected for sequencing amplicons obtained from two cPCR assays based on the 16S rRNA gene, using two sets of primers (fragments of ~800 bp for each protocol) [23]. The samples sequenced for 16S rRNA were also submitted to a cPCR assay based on the 23S rRNA (~900 bp) [24] gene. *Mycoplasma parvum* DNA obtained from a naturally infected swine [25] and ultra-pure sterile water were used as positive and negative controls, respectively.

2.4. Culturing for Bartonella spp.

All blood samples (200 µL) were cultured in 2 mL of *Bartonella*-Alpha Proteobacteria Growth Medium (BAPGM) and were kept under agitation at 37 ◦C with 5% CO2 for 7 days. Then, 200 µL of each enrichment liquid culture containing blood sample were seeded onto a solid culturing medium of enriched chocolate agar [26,27] and maintained under the same conditions for up to four weeks. Colonies suggestive of Bartonellaceae were individually collected, subjected to DNA extraction by the boiling method [28] and subjected to a qPCR assay for *Bartonella* spp., based on the nuoG gene [29]. The remainder of each enrichment liquid culture was also subjected to DNA extraction and qPCR assay. All culture protocols followed Furquim et al. [30].

2.5. Quantitative PCR Assay (qPCR) and Molecular Characterization of Bartonella spp.

The positive samples for the endogenous genes protocols were then submitted to a quantitative screening qPCR for *Bartonella* spp. based on the NADH dehydrogenase gamma subunit (nuoG) gene, as previously described [29]. Serial dilutions were performed to construct standard curves with different concentrations (2.0 × 107 to 2.0 × 100 copies/µL) of the plasmid, which encodes an 83 bp fragment of *Bartonella* henselae nuoG gene (pIDTSMART; Integrated DNA Technologies), diluted in Tris-EDTA. Ultra-pure sterile water was used as a negative control in qPCR assays. The diluted plasmids encoding a fragment of the nuoG gene of *B. henselae* were used as positive controls. Molecular characterization was performed with PCR assays according to Furquim et al. [29]. The number of plasmid copies was determined by the formula (XG/µL DNA/ [Plasmid Length (BP) × 660]) × 6.022 × 1023 × plasmid copies/µL [22]. *Bartonella henselae* DNA from a naturally infected cat was used as a positive control [31]. Ultra-pure sterile water was used as a negative control in cPCR assays.

2.6. Agarose gel Electrophoresis, Sequencing and Phylogenetic Analyses

Results of the cPCR were visualized in 1% agarose gel stained by ethidium bromide solution in a UV transilluminator (ChemiDoc MP Imaging System, Bio Rad®, Hercules, CA, USA), and sequencing of amplicons was performed by Sanger method [32] using ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Waltham, MA, USA). Electropherogram results and consensus sequences were analyzed using Phred-Phrap software version 23 [33]. The BLASTn tool was used to browse and compare with sequences from the GenBank® international database (https://www.ncbi.nlm.nih.gov/genbank/ accessed on 30 October 2022). All sequences obtained in the present study were deposited in GenBank®. For

phylogenetic analyses, 23S rRNA and 16S rRNA (only large) hemoplasma sequences obtained were used. The alignment was performed using MAFFT software (https://mafft.cbrc.jp/alignment/server/ accessed on 30 October 2022), and Bayesian analyses were performed using the CIPRES gateway [34]. The phylogenetic tree edition and rooting (outgroup) were performed using TreeGraph 2.0 beta software [35]. The pairwise distance matrix among sequences of 16S rRNA gene of hemoplasmas detected in the present study was estimated using the Mega-X software version 10.1.8.

2.7. Hematological and Path Analyses

Red blood cells (106/µL), hemoglobin (g/dL), and hematocrit (mm3) were determined using a hematology analyzer device (POCH-iV 100, Syxmex®, São Paulo, Brazil), standardized for ring-tailed coatis according to manufacturers' instructions. Individuals of infected coatis for each hemoplasma genotype detected were expressed by the frequency of occurrence between area (PEP × VBA), sex (Female × Male), and age (Adults × Imatures—puppies and subadults). In order to test possible associations between these variables with the infections, Chi-squared tests were used. To determine the correlation of hemoplasma bacterial load to Anemia indicators, we performed a path analysis using the data obtained from the first capture of each individual (without recaptures), according to Santos et al. [36]. Analyses were performed separately for each hemoplasma genotype. We created the Anemia indicators using red blood cells, hemoglobin, and hematocrit values through a dimensionality reduction by Principal Coordinate Analysis. We used an r-value > 0.60 to interpret the results (positive or negative effect) of the path analysis. We considered the variables statistically significant for p-values < 0.05 for all analyses. All data were analyzed using R software (R Development Core Team, 2015).

3. Results

3.1. DNA Extraction from Coatis' Blood and Ectoparasites Samples and Conventional PCR (PCR) Assays for Endogenous Genes

All DNA extracted from 163 coatis' blood samples were positive in the PCR targeting the gapdh gene and were then submitted to further molecular analyses. On the other hand, 248 out of 265 tick DNA pools were positive at the PCR targeting the 16S rRNA (nine Amblyomma sp. larvae pools, five *A. sculptum* nymphs and three *A. dubitatum* nymphs were negative, and then excluded from the other PCR assays). All 42 lice DNA samples (including pools and individual extractions) were positive in the PCR assay targeting the cox1 endogenous gene and were submitted to further molecular analyses.

1.2. Quantitative PCR Assay (qPCR) for Hemoplasmas

Regarding the first capture, 86/97 (88.6%) coatis were positive in the qPCR for hemoplasmas. When considering captures and recaptures, 146/163 (89.6%) coati blood DNA samples were positive for hemoplasmas. Based on the Tm (Melting Temperature) retrieved from 16S rRNA-based qPCR assay, two different genotypes were detected. A total of 71% (69/97) coatis were positive for a hemoplasma genotype (myc1) presenting Tm = 79–79.5 ◦C; 17% (17/97) individuals were positive for a hemoplasma genotype (myc2) presenting Tm = 77–77.5 ◦C; 12% (11/97) coatis were negative for hemoplasmas. The quantification of positive samples ranged from 2.56×10^0 to 1.26×10^4 copies of a 16S rRNA gene fragment/µL from #myc1 and 6.72×10^0 to 1.41×10^3 from myc2. All quantifications are shown in Board 1—Supplementary File. No double Tm peaks were observed when analyzing individual samples, suggesting, therefore, the absence of coinfections by myc1 and myc2 genotypes in coatis' blood samples. Regarding tick DNA samples, 25/248 (10%) were positive for hemoplasmas [15 *Amblyomma* spp. larvae pools (15/25—60%), two *A. dubitatum* nymph pools (2/25—8%) and eight *A. sculptum* nymph pools (8/25—32%)]. Based on the Tm analysis, the hemoplasma genotype detected in the positive ticks corresponded to myc1. It was not possible to quantify the number of copies of a fragment of the hemoplasma 16S rRNA gene fragment in 21 tick DNA samples due to the low amount of the target DNA (Monte Carlo effect). The quantification of the four positive (one *Amblyomma* sp. larvae pool, two *A. sculptum* nymph pools and two *A. dubitatum* nymph pool) samples ranged from 1.25×10^0 to 2.75×10^0 copies of the hemoplasma 16S rRNA gene fragment/µL (Tm = 79/79.5 ◦C). The 25 positive tick DNA samples were collected from positive coatis presenting a quantification ranging from 3.45×10^1 to 2.02×10^4 copies of the hemoplasma 16S rRNA gene fragment/µL, with Tm of 79/79.5 ◦C (myc1). All 42 lice DNA samples were negative in the qPCR for hemoplasmas. From recaptured animals, two (PEP18 and VBA19) were negative in the qPCR for hemoplasmas in all recaptures. All the other animals presented fluctuations in the estimated hemoplasma bacterial load, albeit no pattern of bacteria load was observed during recaptures (Table 1). Based on the Tm analysis, only one hemoplasma genotype was found infecting coatis in different recapture times.

Table 1. Identification of coatis (*Nasua nasua*) sampled between 2018–2019 in two peri-urban areas from Campo Grande do Sul, Mato Grosso do Sul, Brazil, with identification of the animal (ID), sex, qPCR melting temperature (Tm), detected hemoplasma genotype (myc1 with Tm = 79/79.5 ◦C or myc2 with Tm = 77–77.5 ◦C), and quantification of the detected hemoplasma genotype based on qPCR assay based on the 16S rRNA gene during successive recaptures.

ID	Sex	1C	2C	3C	4C	5C	6C
PEP01	M	$2.02x10^4$ (Tm=79.5) myc1	$2.66x10^2$ (Tm=79.5) myc1	$1.18x10^3$ (Tm= 79.5) myc1	x	x	x
PEP02	M	$6.97x10^2$ (Tm=79.5) myc1	$8.76x10^2$ (Tm=79.5) myc1	x	x	x	x
PEP04	M	Negative	$6.83x10^2$ (Tm=79.5) myc1	$3.80\ x10^3$ (Tm=79.5) myc1	x	x	x
PEP05	M	$5.28x10^1$ (Tm=77.5) myc2	$3.43x10^2$ (Tm=77.5) myc2	x	x	x	x
PEP12	F	$1.65x10^2$ (Tm=79/79.5) myc1	$4.09x10^2$ (Tm=79/79.5) myc1	x	x	x	x
PEP17	F	$2.86x10^1$ (Tm=79/79.5) myc1	$1.83x10^2$ (Tm=79/79.5) myc1	x	x	x	x
PEP18	F	Negative	Negative	x	x	x	x
PEP20	F	$1.97x10^2$ (Tm=79/79.5) myc1	$3.63x10^2$ (Tm=79/79.5) myc1	x	x	x	x
PEP23	F	$1.89x10^2$ (Tm=79.5) myc1	$1.36x10^3$ (Tm=79.5) myc1	x	x	x	x
PEP24	F	$1.12x10^2$ (Tm=79/79.5) myc1	$1.65x10^2$ (Tm=79/79.5) myc1	x	x	x	x
PEP31	M	$1.38x10^3$ (Tm=79.5) myc1	$4.66x10^3$ (Tm=79.5) myc1	x	x	x	x
PEP32	M	$1.61x10^3$ (Tm=79.5) myc1	$1.04x10^3$ (Tm=79.5) myc1	x	x	x	x
PEP43	M	$6.30x10^2$ (Tm=79.5) myc1	$1.58x10^3$ (Tm=79.5) myc1	x	x	x	x

VBA01	M	$2.32x10^2$ (Tm=79/79.5) myc1	$1.36x10^1$ (Tm=79/79.5) myc1	x	x	x	x
VBA03	F	$5.02x10^1$ (Tm=79.5) myc1	$3.95x10^2$ (Tm=79.5) myc1	$2.12x10^2$ (Tm=79.5) myc1	$3.49x10^2$ (Tm=79.5) myc1	$1.55x10^2$ (Tm=79.5) myc1	$5.39\ x10^1$ (Tm=79.5) myc1
VBA05	M	$4.00x10^2$ (Tm=79.5) myc1	$1.70x10^2$ (Tm=79.5) myc1	x	x	x	x
VBA06	M	$1.62x10^2$ (Tm=79.5) myc1	$5.74x10^2$ (Tm=79.5) myc1	x	x	x	x
VBA07	M	$3.18x10^2$ (Tm=79.5) myc1	$3.52x10^2$ (Tm=79.5) myc1	$2.90x10^2$ (Tm=79.5) myc1	$2.96x10^2$ (Tm=79.5) myc1	x	x
VBA08	F	$2.25x10^2$ (Tm=79.5) myc1	$5.49x10^1$ (Tm=79.5) myc1	$8.98x10^2$ (Tm=79.5) myc1	x	x	x
VBA09	M	Negative	Negative	Negative	Negative	x	x
VBA10	F	$1.05x10^3$ (Tm=77.5) myc2	$6.79x10^1$ (Tm=77.5) myc2	$7.16x10^1$ (Tm=77.5) myc2	x	x	x
VBA11	M	Negative	$1.88x10^4$ (Tm=79/79.5) myc1	$7.11x10^3$ (Tm=79/79.5) myc1	x	x	x
VBA12	F	$9.60x10^0$ (Tm=79) myc1	$7.47x10^0$ (Tm=79) myc1	x	x	x	x
VBA16	F	$5.16x10^2$ (Tm=77.5) myc2	$1.31x10^1$ (Tm=77.5) myc2	$2.62x10^1$ (Tm=77.5) myc2	$1.10x10^2$ (Tm=77.5) myc2	x	x
VBA17	F	$8.10x10^3$ (Tm=79.5) myc1	$9.79x10^1$ (Tm=79.5) myc1	x	x	x	x
VBA19	F	$2.51x10^2$ (Tm=79.5) myc1	$5.48x10^1$ (Tm=79.5) myc1	x	x	x	x
VBA21	M	$3.59x10^0$ (Tm=77/77.5) myc2	$5.64x10^0$ (Tm=77/77.5) myc2	$3.00x10^2$ (Tm=77/77.5) myc2	$4.94x10^0$ (Tm=77/77.5) myc2	$2.85x10^0$ (Tm=77/77.5) myc2	$5.98x10^4$ (Tm=77/77.5) myc2
VBA22	F	$5.14x10^0$	$5.39x10^0$	x	x	x	x

		(Tm=79) myc1	(Tm=79) myc1				
VBA23	F	$7.45x10^0$ (Tm=77.5) myc2	$7.45x10^0$ (Tm=77.5) myc2	x	x	x	x
VBA25	M	Negative	Negative	$1.70\ x10^1$ (Tm=79) myc1	$3.33x10^0$ (Tm=79) myc1	$9.03x10^3$ (Tm=79.5) myc1	x
VBA29	M	$2.18x10^2$ (Tm=79/79.5) myc1	$4.65x10^2$ (Tm=79/79.5) myc1	x	x	x	x
VBA38	F	$6.33x10^0$ (Tm=79) myc1	$3.18x10^0$ (Tm=79) myc1	x	x	x	x
VBA41	F	$2.05x10^3$ (Tm=79/79.5) myc1	$2.33x10^2$ (Tm=79/79.5) myc1	x	x	x	x
VBA44	M	$2.09x10^2$ (Tm=77.5) myc1	$2.39x10^2$ (Tm=77.5) myc1	$1.88\ x10^2$ (Tm=77.5) myc1	x	x	x
VBA55	M	$2.03x10^2$ (Tm=79) myc1	$2.09x10^2$ (Tm=79) myc1	x	x	x	x

PEP: Parque Estadual do Prosa; VBA: Vila da Base Aérea; M: Male; F: Female; 1C: First capture; 2C: Second capture; 3C: Third capture; 4C: Fourth capture; 5C: Fifth capture; 6C: Sixth capture; x: non-captured

3.3. Molecular characterization of hemoplasmas

Fifteen samples (seven from PEP and eight from VBA) were randomly chosen for sequencing of both 16S rRNA gene fragments, resulting in sequences that ranged from 646 to 1,124 base pairs (bp) (GenBank® Acession numbers: OP964769 – OP964810). Phylogenetic analyses (Bayesian method) clustered the sequences detected in the present study into two clades: while the clade #1 was composed by myc1 (detected in the present study), hemoplasama previously detected in coatis from Pantanal (Mato Grosso do Sul state), and '*Candidatus* M. haematonasua', previously detected in coatis from Iguaçu National Park (Paraná state), clade #2 was composed by myc2 (detected in the present study) and *Mycoplasma haemofelis* and *Mycoplasma haemocanis* (**Figure 1**). The different lengths of the nucleotide sequences did not affect the phylogenetic analysis result. While

myc1 was represented by amplicons showing Tm=79-79.5ºC, myc2 was represented by amplicons showing Tm=77-77.5ºC.

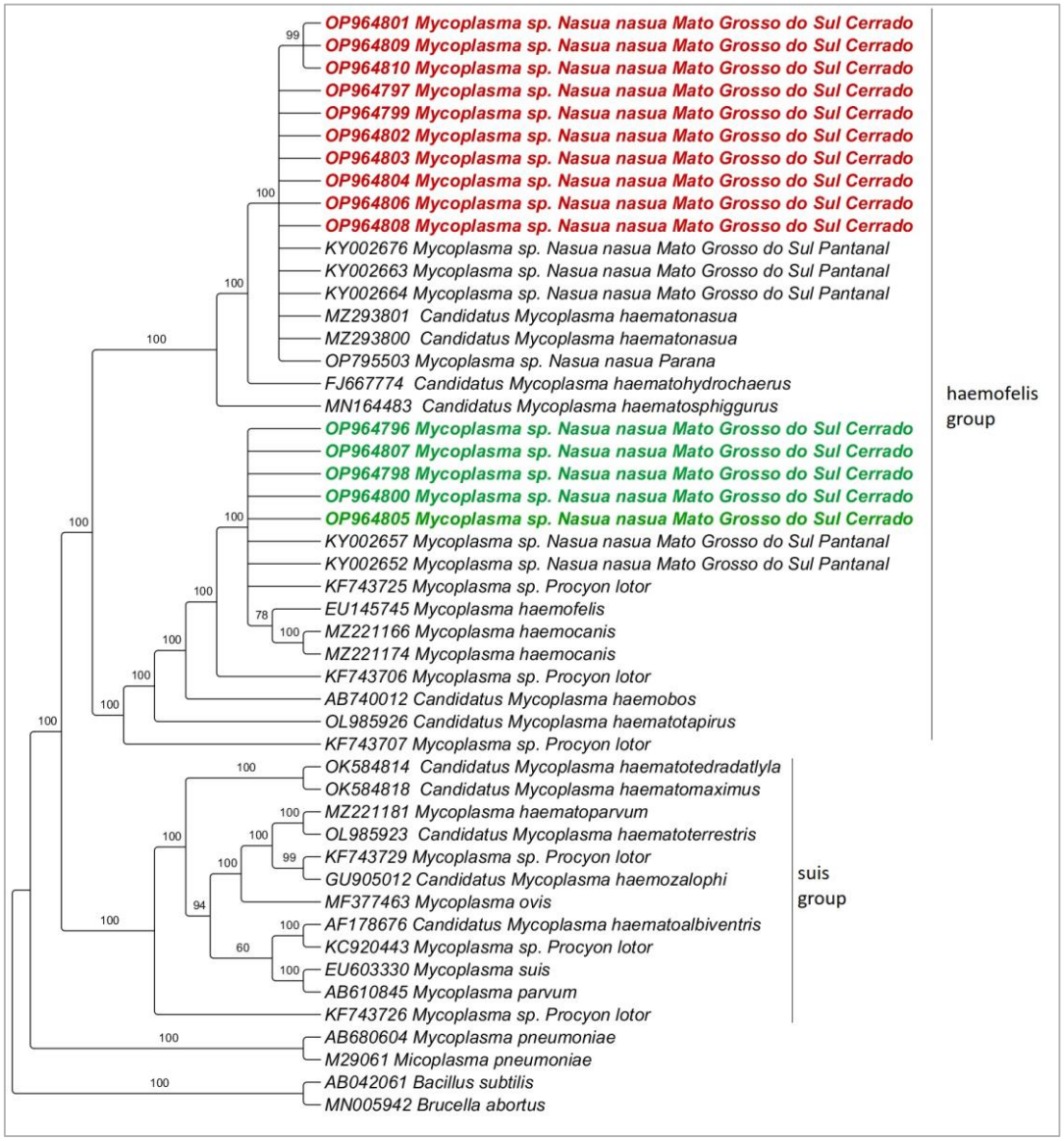

Figure 1. Phylogenetic tree based on Bayesian method of an alignment of 1,252 bp and GTR+G evolutionary model of the 16S rRNA gene of hemoplasmas detected in coati blood samples from urban forested fragments of Campo Grande city, Mato Grosso do Sul state, Brazil. *Bacillus subtilis* (AB042061) and *Brucella abortus* (MN005942) were used as outgroups. Numbers at the nodes correspond to posterior probabilities. Sequences detected in the present study were highlighted in red bold (myc 1) and in green bold (myc 2).

Regarding 23S rRNA gene, sequences ranging from 724 to 764 bp were obtained (GenBank® Acession numbers: OP886219 - OP886233). Phylogenetic analyses (Bayesian method) clustered the sequences detected in the present study into one large clade composed by two sub-clades: while one sub-clade was composed by the myc1 detected in the present study and '*Candidatus* Mycoplasma haematonasua', '*Candidatus* Mycoplasma haematosphiggurus' and '*Candidatus* Mycoplasma haematohydrochaerus', the second sub-clade was composed by myc2 detected in the present study (**Figure 2**).

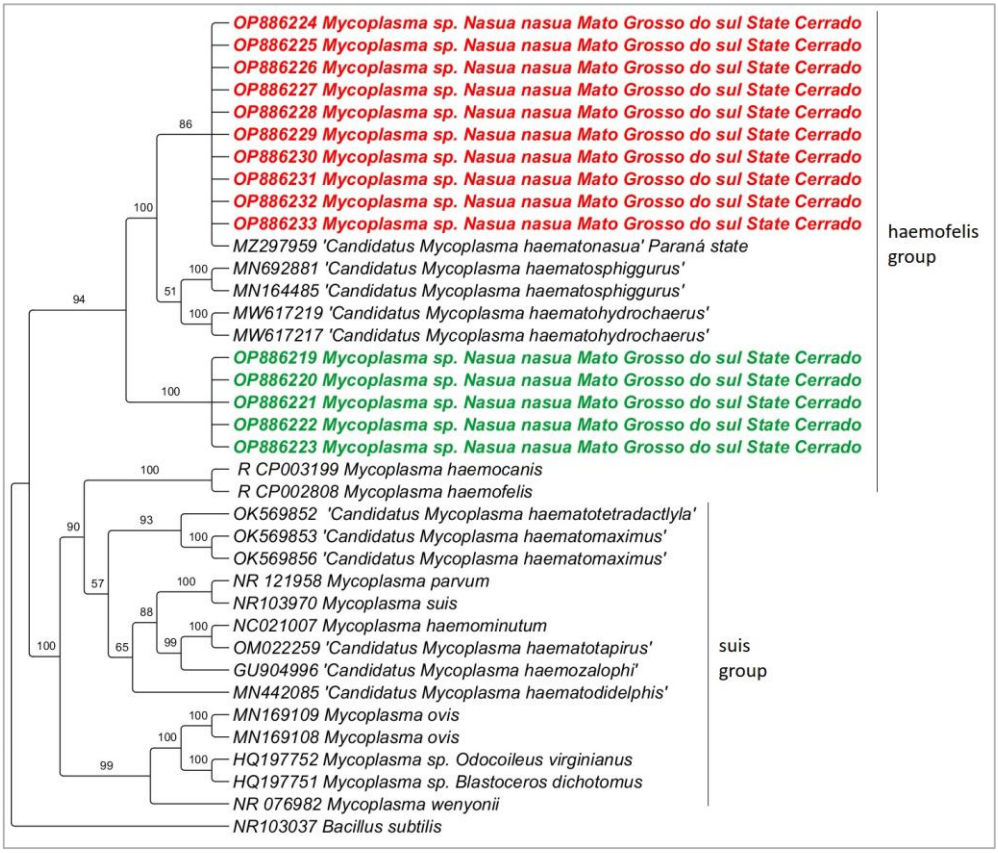

Figure 2. Phylogenetic tree based on Bayesian method of an alignment of 890 bp and TN+G evolutionary model of the 23S rRNA gene of hemoplasmas detected in coati blood samples from urban fragments areas of Campo Grande city, Mato Grosso do Sul state, Brazil. *Bacillus subtilis* (NR103037) was used as outgroup. Numbers at the nodes correspond to posterior probabilities. Sequences detected in the present study were highlighted in red bold (myc1) and in green bold (myc2).

Based on the 16S rRNA, the pairwise distance between myc1 genotype and '*Candidatus* M. haematonasua' ranged from 0 to 0.1%; genotypes myc1 and myc2 showed genetic divergence ranging from 0 to 1.3%; myc2 genotype and *Mycoplasma* sp. from *Procyon lotor* showed genetic divergence of 0; when comparing myc2 genotype and *M. haemofelis* and *M. haemocanis*, genetic divergence from 0.005 to 0.006 was found.

3.4. Culturing and qPCR assay (qPCR) for Bartonella *spp.*

None of the 163 coatis' blood samples were positive neither at the qPCR assay for *Bartonella* spp. from blood DNA samples nor at liquid and solid cultures followed by qPCR. All *N. pallidus* DNA samples were negative in the qPCR for *Bartonella* spp. Four tick samples were positive in the qPCR for *Bartonella* spp.: two *Amblyomma* sp. larvae pools (one from PEP and one from VBA; Cq values of 37.2 and 38.9) and two *A. dubitatum* nymph pools (one from PEP and one from VBA; Cq values of 36.9 and 38.5). The quantification of the number of copies of a fragment of the *Bartonella nuoG* gene fragment was not possible due to Monte-Carlo effect. None tick DNA sample positive in the qPCR was positive in the conventional PCR assays for *Bartonella* sp. targeting the *gltA*, *rpoB*, *ftsZ*, *pap-31*, *ribC*, and *nuoG* molecular markers.

3.5. Hematological analyses and path analysis

All hematological analyses are shown in Board 2 – Supplementary file. Our analysis showed no association between the analyzed variables (area, sex, and age) and the positivity for myc1 and myc2. Regarding area, the occurrence of coatis positive to myc1 were 81% (34/42) in PEP and 64% (35/55) in VBA (Chi^2= 2.68, *p*= 0.10). On the other hand, 9% (04/42) were positive to myc2 in PEP and 24% (13/55) in VBA (Chi^2= 2.38, *p*= 0.12). The presence of DNA of myc1 was identified in 75% (42/56) females and 66% (27/41) males (Chi^2= 0.57, *p*= 0.45). On the other hand, myc2 was detected in 18% (10/56) females and 17% (7/41) males (Chi^2= 1.10, *p*= 1). Lastly, 77% (54/70) adults and 55% (15/27) imatures (puppies and subadults) were positive to myc1 (Chi^2= 3.43, *p*= 0.06), and 14% (10/70) and 26% (7/27) adults and subadults, respectively, were positive to myc2 (Chi^2= 1.11, *p*=0.29). Furthermore, there was also no association between hemoplasma bacterial load (CMH: *p*=0. 89, CMP: *p*=0.81) and the anemia indicators for any of the hemoplasma species (myc1 *p*=0. 89; myc2 *p*= 0.67).

2. Discussion

Herein, the high rates of coatis positive for hemoplasmas corroborate previous studies performed in Brazil. For instance, Sousa et al. [4] found 77.4% coatis positive for hemoplasmas in the Brazilian Pantanal, with two different genotypes: one closely related to the capybara (*Hydrochoerus hydrochaeris*)-associated hemoplasma in Brazil, and another one closely related to *Mycoplasma haemofelis*. In the present study, a similar result was

observed, with the formation of two different clades with two distinct detected sequences. Interestingly, the melting temperature (Tm) allowed the differentiation between the two hemoplasma genotypes detected in the present study. Therefore, the pan-hemoplasma qPCR based on the 16S rRNA can be a good tool to screen coatis' blood and ectoparasite samples in areas where more than one hemoplasma genotype circulates in coatis.

Even though the main route of transmission of hemoplasmas is still unknown, alternative routes of transmission have been demonstrated, such as vertical, blood transfusion, and aggressive interactions among cats and wild rodents [37–41]. Sousa et al. [4] pointed out that other routes of transmission, such as agonistic encounters between individuals from different groups and inside the same group, might have more importance in the transmission of hemoplasmas among these animals, mainly due to the gregarious behavior of this mammal species, with the formation of large familiar groups [42]. The positivity for hemoplasmas among immature coatis (55%) might also suggest the possibility of vertical transmission of hemoplasmas among coatis, which should be further investigated in the future.

Regarding recaptured animals, we observed a fluctuation in the estimated hemoplasma bacterial load among the successful recaptures, although we could not observe a pattern of such fluctuation of quantification. While some animals presented constant hemoplasma bacterial load throughout the recaptures, others showed an increase or decrease in bacterial load. The lack of pattern on the estimated quantification of hemoplasmas might be due to the non-standardization among samplings dates since all recaptures were performed by chance. Based on Tm analysis, all recaptured animals were infected with the same hemoplasma genotypes, and no co-infection of myc1 and myc2 was observed.

In the present study, no association was found between hemoplasma bacterial load (estimate quantification of a fragment of the 16S rRNA gene of hemoplasmas by qPCR) and red blood cell parameters. According to the results found herein, the infection by myc1 and myc2 seems not to cause anemia in wild coatis. The impact of these infections on coatis kept in captivity and under stressful conditions should be investigated in the future. It is known that some hemoplasmas species that infect domestic animals (e.g., *Mycoplasma suis, Mycoplasma haemocanis*, *Mycoplasma haemofelis*) can cause moderate to severe disease in their hosts [4]. Several studies have indicated that hemoplasma infection can lead to a decrease in red blood cell parameters [4,43–45], which can be associated with bacterial load [46]. On the other hand, some studies did not find such an association, which can be due to

the involved or the presence of chronic carriers, with no red blood cell abnormalities among the tested animals [2,47].

Despite the efforts of a multi-approach diagnostic workflow, *Bartonella* sp. was not detected in coati blood samples. Interestingly, *Bartonella* spp. DNA was detected by qPCR in 10% of ticks collected from negative coatis. The tick samples positive in the qPCR assay for *Bartonella* spp. were negative in cPCR based on six molecular markers, likely because of the low amount of the targeted DNA, which might be below the limit of the detection of the conventional PCR assays used for molecular characterization. Transmission of *Bartonella* species occurs mainly through fleas and lice [48,49]. The positivity of *A. dubitatum* nymphs found in the present study might be associated with the feeding of a Bartonellaceae-infected host in the larval stage. Interestingly, *Bartonella* DNA was detected in larvae pools collected from negative coatis. Due to the low quantity of DNA copies in ticks, the detection of *Bartonella* DNA in nymphs and larvae might merely indicate the presence of remnant blood with *Bartonella* sp. DNA from a previous blood meal, without any involvement of this tick species in *Bartonella* sp. transmission. The role of *Amblyomma* ticks in the transmission and maintenance of *Bartonella* spp. should be further investigated by experimental studies.

3. Conclusions

Hemoplasmas are endemic in coatis from urban areas in Midwestern Brazil, with the presence of two hemoplasma genotypes. The infection of wild coatis by the two hemoplasma genotypes was not associated with anemia. *Amblyomma* spp. ticks seem not to play an important role in the transmission of hemoplasmas among coatis in the two studied areas. *Bartonella* spp. seems not to occur in wild coatis parasitized by *Amblyomma* ticks and *N. pallidulus* louse in forested urban areas in midwestern Brazil.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens12040538/s1, Table S1. Identification of coatis sampled and recaptured in the two locations (PEP and VBA), field number, sex, age group and qPCR positivity for hemoplasmas based on the 16S rRNA gene. The quantification cycle average values (Cq), quantification average (number of copies of a 16S rRNA gene fragment per µL), melting temperature (Tm), and positivity in conventional PCR assays for two different regions of the 16S RNA gene, named here as “first” (900 bp) and “second” (900 pb) fragments, and samples selected for sequencing an 800 bp fragment of the 23S rRNA gene.

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Institutional Review Board Statement: All methods were carried out in accordance with relevant guidelines and regulations and were approved by the “Instituto Chico Mendes de Biodiversidade” (ICMBio) (SISBIO 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB 001/2018) and ‘Sistema Nacional de Gestão de Patrimônio Genético’ (ABDF0B5).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated and analyzed during the current study are available in the NCBI GenBank® Nucleotide platform and can be accessed through accession numbers: OP964769–OP964810 for 16SrRNA gene and OP886219–OP886233 for 23SrRNA gene.

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Supplementary file

Assessment of hemoplasma and *Bartonella* spp. bacteremia on naturally infected coatis (*Nasua nasua*) and associated ectoparasites, and their influence on hematological parameters

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Board 1. Identification of coatis sampled and recaptured in the two locations (PEP and VBA), field number, sex, age group and qPCR positivity for hemoplasmas based on the 16S rRNA gene. The quantification cycle average values (Cq), quantification average (number of copies of a 16S rRNA gene fragment per µL), melting temperature (Tm), positivity in conventional PCR assays for two different regions of the 16S RNA gene, named here as "first" (900pb) and "second" (900pb) fragments, and samples selected for sequencing an 800 bp fragment of the 23S rRNA gene.

Collection date	Identification number	Localization	Sex	Age	Mean Cq	Mean Quantification of Copies of a Gene Fragment 16S rRNA/µL	Melting temperature	1° fragment 16S rRNA (900pb) (Maggi et al. 2013)	2° 1fragment (900 pb) (Maggi et al. 2013)	23SrRNA (800 pb) (Mongruel et al. 2020)

13/03/2018	PEP 02	PEP[1]	M	Adulto	21,55	$6,97x10^2$	79,5	+	+	x
14/03/2018	PEP 04	PEP	M	Adulto	Negative	Negative	-	-	-	x
15/03/2018	PEP 06	PEP	F	Adulto	22,48	$3,84x10^2$	79	Sequenced	Sequenced	Sequenced
15/03/2018	PEP 08	PEP	F	Adulto	23,47	$1,62x10^2$	79,5	+	+	x
16/03/2018	PEP 10	PEP	M	Adulto	27,46	$1,57x10^1$	79,5	+	+	x
16/03/2018	PEP 11	PEP	F	Adulto	22,85	$3,03x10^2$	79	+	+	x
20/03/2018	PEP 13	PEP	M	Subadulto	18,41	$5,27x10^3$	79,5	+	+	x
21/03/2018	PEP 14	PEP	M	Adulto	26,24	$3,45x10^1$	79	+	+	x
21/03/2018	PEP 15	PEP	F	Adulto	25,92	$4,21x10^1$	79/79,5	+	+	x
22/03/2018	PEP 17	PEP	F	Adulto	26,53	$2,86\ x10^1$	79,5	+	+	x
30/04/2018	VBA 01	VBA[2]	M	Adulto	23,27	$2,32x10^2$	79,5	+	+	x
02/05/2018	VBA 03	VBA	F	Adulto	21,75	$5,02\ x10^1$	79,5	+	+	x
02/05/2018	VBA 04	VBA	F	Adulto	25,45	$5,75x10^1$	79,5	+	+	x
02/05/2018	VBA 05	VBA	M	Adulto	22,09	$4,00x10^2$	79,5	+	+	x
02/05/2018	VBA 06	VBA	M	Adulto	23,47	$1,62x10^2$	79	+	+	x
03/05/2018	VBA 07	VBA	M	Adulto	22,44	$3,18x10^2$	79,5	+	+	x
04/05/2018	VBA 08	VBA	F	Adulto	23,32	$2,25x10^2$	79,5	+	+	x
04/05/2018	VBA 09	VBA	M	Subadulto	Negative	Negative	-	-	-	x
04/05/2018	VBA 10	VBA	F	Adulto	20,92	$1,05x10^3$	77,5	+	+	x
07/05/2018	VBA 11	VBA	M	Adulto	Negative	Negative	-	-	-	x
09/05/2018	VBA 12	VBA	F	Adulto	28,23	$9,60x10^0$	79	-	+	x
10/05/2018	VBA 13	VBA	M	Adulto	21,32	$8,10x10^2$	79,5	+	+	x
28/05/2018	PEP 18	PEP	F	Filhote	Negative	Negative	-	-	-	x
28/05/2018	PEP 19	PEP	F	Filhote	25,57	$5,36\ x10^1$	77,5	+	-	x
29/05/2018	PEP 20	PEP	F	Adulto	23,52	$1,97x10^2$	79,5	+	+	x
29/05/2018	PEP 21	PEP	F	Adulto	25,71	$4,82\ x10^1$	79,5	+	+	x
29/05/2018	PEP 22	PEP	F	Adulto	23,6	$1,87x10^2$	79,5	+	+	x
30/05/2018	PEP 02B	PEP	M	Adulto	21,2	$8,76x10^2$	79,5	+	+	x
30/05/2018	PEP 01B	PEP	M	Adulto	16,12	$2,02x10^4$	79,5	+	+	x
30/05/2018	PEP 03B	PEP	F	Adulto	22,67	$3,41x10^2$	79,5	+	+	x
30/05/2018	PEP 05B	PEP	M	Adulto	25,17	$5,28x10^1$	77,5	Sequenced	Sequenced	Sequenced

30/05/2018	PEP 23	PEP	F	Adulto	23,59	$1,89x10^2$	79,5	Sequenced	Sequenced	Sequenced
11/06/2018	PEP 12B	PEP	F	Adulto	23,44	$1,65x10^2$	79	+	+	x
12/06/2018	PEP 24	PEP	F	Adulto	24,4	$1,12x10^2$	79	+	+	x
12/06/2018	PEP 25	PEP	M	Adulto	17,24	$1,11x10^4$	79,5	+	+	x
15/06/2018	PEP 04B	PEP	M	Adulto	21,59	$6,83x10^2$	79,5	+	+	x
15/06/2018	PEP 17B	PEP	F	Adulto	23,64	$1,83x10^2$	79	+	+	x
18/06/2018	VBA 03B	VBA	F	Adulto	22,26	$3,95x10^2$	79,5	Sequenced	Sequenced	Sequenced
19/06/2016	VBA 15	VBA	F	Adulto	18,41	$5,27\ x10^3$	79,5	+	+	x
19/06/2016	VBA 12B	VBA	F	Adulto	28,63	$7,47x10^0$	79	-	+	x
19/06/2016	VBA 08B	VBA	F	Adulto	25,11	$5,49\ x10^1$	79,5	+	+	x
20/06/2018	VBA 10B	VBA	F	Adulto	24,79	$6,79\ x10^1$	77,5	+	+	x
20/06/2018	VBA 16	VBA	F	Adulto	21,7	$5,16x10^2$	77,5	+	+	x
20/06/2018	VBA 17	VBA	F	Adulto	17,51	$8,10\ x10^3$	79,5	+	+	x
20/06/2018	VBA 11B	VBA	M	Adulto	16,23	$1,88x10^4$	79	Sequenced	Sequenced	Sequenced
21/06/2018	VBA 18	VBA	F	Adulto	20,3	$1,30\ x10^3$	77,5	+	+	x
21/06/2018	VBA 19	VBA	F	Adulto	22,8	$2,51x10^2$	79,5	+	+	x
22/06/2018	VBA 20	VBA	M	Adulto	25,08	$5,61\ x10^1$	79,5	+	+	x
25/06/2018	VBA 21	VBA	M	Filhote	29,26	$3,59x10^0$	77	-	-	x
25/06/2018	VBA 07B	VBA	M	Adulto	22,29	$3,52x10^2$	79,5	+	+	x
26/06/2018	VBA 22	VBA	F	Filhote	28,71	$5,14x10^0$	79	-	-	x
26/06/2018	VBA 23	VBA	F	Adulto	28,16	$7,45x10^0$	77,5	+	-	x
26/06/2018	VBA 24	VBA	M	Subadulto	28,56	$5,71x10^0$	79	-	-	x
26/06/2018	VBA 09B	VBA	M	Subadulto	Negative	Negative	-	-	-	x
26/06/2018	VBA 25	VBA	M	Filhote	Negative	Negative	-	-	-	x
29/06/2018	VBA 26	VBA	F	Adulto	28,61	$5,51x10^0$	77	-	-	x
30/07/2018	PEP 23B	PEP	F	Adulto	20,24	$1,36\ x10^3$	79,5	Sequenced	Sequenced	Sequenced
30/07/2018	PEP 04C	PEP	M	Adulto	18,66	$3,80\ x10^3$	79,5	+	+	x
31/07/2018	PEP 27	PEP	F	Adulto	21,66	$5,29x10^2$	79,5	+	+	x
31/07/2018	PEP 28	PEP	M	Adulto	23,22	$1,90x10^2$	77,5	+	+	x
31/07/2018	PEP 29	PEP	F	Adulto	22,1	$3,97x10^2$	79,5	Sequenced	Sequenced	Sequenced
31/07/2018	PEP 30	PEP	F	Adulto	23,03	$2,16x10^2$	79,5	+	+	x

31/07/2018	PEP 03C	PEP	F	Adulto	21,37	$6,58x10^2$	79,5	+	+	x
31/07/2018	PEP 20B	PEP	F	Adulto	22,25	$3,63x10^2$	79	-	+	x
01/08/2018	PEP 31	PEP	M	Adulto	20,21	$1,38\ x10^3$	79,5	+	+	x
01/08/2018	PEP 05C	PEP	M	Adulto	22,33	$3,43x10^2$	77.5	+	+	x
02/08/2018	PEP 24B	PEP	F	Adulto	23,45	$1,65x10^2$	79/79,5	+	+	x
02/08/2018	PEP 32	PEP	M	Adulto	19,97	$1,61x10^3$	79,5	+	+	x
02/08/2018	PEP 33	PEP	M	Adulto	23,14	$2,06x10^2$	79	+	+	x
03/08/2018	PEP 34	PEP	F	Adulto	25,72	$3,68\ x10^1$	79	+	+	x
03/08/2018	PEP 35	PEP	F	Adulto	Negative	Negative	-	-	-	x
03/08/2018	PEP 36	PEP	F	Subadulto	26,48	$1,13\ x10^1$	77,5	+	-	x
06/08/2018	PEP 12C	PEP	F	Adulto	21,42	$4,09x10^2$	79,5	+	+	x
06/08/2018	PEP 37	PEP	F	Adulto	16,59	$1,26x10^4$	79,5	+	+	x
06/08/2018	PEP 38	PEP	F	Adulto	21,61	$3,57x10^2$	79,5	+	+	x
07/08/2018	PEP 39	PEP	M	Adulto	25,08	$3,04\ x10^1$	79	+	-	x
08/08/2018	PEP 40	PEP	F	Adulto	24,04	$6,38\ x10^1$	79	+	-	x
08/08/2018	PEP 42	PEP	F	Adulto	21	$5,55x10^2$	79,5	+	+	x
08/08/2018	PEP 01C	PEP	M	Adulto	22,03	$2,66x10^2$	79,5	+	+	x
09/08/2018	PEP 43	PEP	M	Adulto	20,88	$6,30x10^2$	79,5	+	+	x
09/08/2018	PEP 26B	PEP	M	Adulto	21,54	$3,76x10^2$	79	+	+	x
20/08/2018	VBA 10C	VBA	F	Adulto	23,88	$7,16\ x10^1$	77,5	+	+	x
20/08/2018	VBA 27	VBA	M	Adulto	Negative	Negative	-	-	-	x
21/08/2018	VBA 07C	VBA	M	Adulto	21,9	$2,90x10^2$	79,5	+	+	x
24/08/2018	VBA 28	VBA	F	Adulto	20,58	$7,46x10^2$	79,5	+	+	x
24/08/2018	VBA 16B	VBA	F	Adulto	26,27	$1,31\ x10^1$	77,5	+	-	x
24/08/2018	VBA 21B	VBA	M	Filhote	27,46	$5,64x10^0$	77,5	+	+	x
24/08/2018	VBA 30	VBA	F	Filhote	25,93	$1,68\ x10^1$	79,5	+	+	x
27/08/2018	VBA 31	VBA	F	Adulto	23,29	$1,08x10^2$	79,5	+	+	x
27/08/2018	VBA 32	VBA	F	Adulto	Negative	Negative	-	-	-	x
27/08/2018	VBA 25B	VBA	M	Filhote	Negative	Negative	-	-	-	x
28/08/2018	VBA 33	VBA	F	Adulto	25,54	$2,32\ x10^1$	77,5	+	+	x
28/08/2018	VBA 34	VBA	M	Subadulto	22,36	$2,17x10^2$	77,5	+	+	x

28/08/2018	VBA 35	VBA	M	Subadulto	25,55	$2,21 \times 10^{1}$	79,5	+	+	x
28/08/2018	VBA 36	VBA	M	Filhote	25,29	$2,62 \times 10^{1}$	76	+	-	x
28/08/2018	VBA 03C	VBA	F	Adulto	22,35	$2,12 \times 10^{2}$	79,5	Sequenced	Sequenced	Sequenced
29/08/2018	VBA 37	VBA	F	Adulto	22,43	$2,08 \times 10^{2}$	77,5	+	+	x
30/08/2018	VBA 38	VBA	F	Filhote	27,3	$6,33 \times 10^{0}$	79	+	-	x
30/08/2018	VBA 22B	VBA	F	Filhote	27,53	$5,39 \times 10^{0}$	79	+	-	x
30/08/2018	VBA 09C	VBA	M	Adulto	Negative	Negative	-	-	-	x
31/08/2018	VBA 40	VBA	F	Adulto	21,87	$3,03 \times 10^{2}$	79,5	+	+	x
01/10/2018	PEP 18B	PEP	F	Filhote	Negative	Negative	-	-	-	x
02/10/2018	PEP 44	PEP	M	Filhote	26,99	$7,88 \times 10^{0}$	79,5	+	-	x
02/10/2018	PEP 45	PEP	M	Filhote	20,18	$9,85 \times 10^{2}$	79,5	+	+	x
02/10/2018	PEP 46	PEP	F	Filhote	28	$3,93 \times 10^{0}$	79	+	-	x
02/10/2018	PEP 47	PEP	M	Filhote	27,04	$7,78 \times 10^{0}$	79	+	-	x
03/10/2018	PEP 31B	PEP	M	Adulto	18	$4,66 \times 10^{3}$	79,5	-	+	x
04/10/2018	PEP 48	PEP	F	Adulto	23,54	$9,13 \times 10^{1}$	77	+	-	x
04/10/2018	PEP 32B	PEP	M	Adulto	20,12	$1,04 \times 10^{3}$	79,5	+	+	x
04/10/2018	PEP 49	PEP	M	Filhote	Negative	Negative	-	-	-	x
08/10/2018	PEP 01D	PEP	M	Adulto	19,93	$1,18 \times 10^{3}$	79,5	+	+	x
09/10/2018	PEP 43B	PEP	M	Adulto	19,32	$1,58 \times 10^{3}$	79,5	+	+	x
09/10/2018	PEP 50	PEP	F	Adulto	17,55	$5,49 \times 10^{3}$	79,5	+	+	x
10/10/2018	PEP 51	PEP	M	Adulto	17,62	$5,22 \times 10^{3}$	79,5	+	+	x
22/10/2018	VBA 07D	VBA	M	Adulto	21,66	$2,96 \times 10^{2}$	79,5	+	+	x
23/10/2018	VBA 09D	VBA	M	Adulto	Negative	Negative	-	-	-	x
23/10/2018	VBA 11C	VBA	M	Adulto	17,19	$7,11 \times 10^{3}$	79,5	+	+	x
23/10/2018	VBA 03D	VBA	F	Adulto	21,45	$3,49 \times 10^{2}$	79,5	Sequenced	Sequenced	Sequenced
29/10/2018	VBA 21C	VBA	M	Subadulto	21,65	$3,00 \times 10^{2}$	77	+	+	x
31/10/2018	VBA 39B	VBA	F	Adulta	24,13	$5,14 \times 10^{1}$	77,5	+	-	x
31/10/2018	VBA 23B	VBA	F	Adulta	27,49	$4,74 \times 10^{0}$	77,5	+	-	x
31/10/2018	VBA 29B	VBA	M	Filhote	22,09	$2,18 \times 10^{2}$	79	+	+	x
01/11/2018	VBA 41	VBA	F	Adulto	18,94	$2,05 \times 10^{3}$	79,5	+	+	x
06/11/2018	VBA 42	VBA	M	Filhote	23,49	$8,13 \times 10^{1}$	77,5	+	+	x

06/11/2018	VBA 25C	VBA	M	Filhote	25,69	1,70 x10^1	79	+	+	x
06/11/2018	VBA 43	VBA	M	Adulto	28,35	2,56x10^0	79	-	-	x
07/11/2018	VBA 06B	VBA	M	Adulto	20,73	5,74x10^2	79	+	+	x
07/11/2018	VBA 44	VBA	M	Adulto	22,16	2,09x10^2	77,5	+	+	x
21/01/2019	VBA 16C	VBA	F	Adulto	25,08	2,62 x10^1	77,5	+	+	x
21/01/2019	VBA 39C	VBA	F	Adulto	Negative	Negative	-	-	-	x
21/01/2019	VBA 41B	VBA	F	Adulto	22	2,33x10^2	79	+	+	x
22/01/2019	VBA 45	VBA	F	Adulto	Negative	Negative	-	-	-	x
22/01/2019	VBA 29C	VBA	M	Subadulto	21,02	4,65x102	79,5	+	+	x
22/01/2019	VBA 46	VBA	F	Filhote	20,83	5,33x10^2	79,5	+	+	x
23/01/2019	VBA 47	VBA	F	Adulto	21,66	2,96x10^2	77,5	+	+	x
23/01/2019	VBA 48	VBA	F	Adulto	25,55	1,88 x10^1	79	+	+	x
23/01/2019	VBA 03E	VBA	F	Adulto	22,58	1,55x10^2	79,5	+	+	x
23/01/2019	VBA 49	VBA	F	Adulto	23,41	1,02x10^2	79,5	+	+	x
23/01/2019	VBA 05D	VBA	M	Adulto	22,44	1,70x10^2	79,5	+	+	x
29/01/2019	VBA 17B	VBA	F	Adulto	23,22	9,79 x10^1	79,5	+	+	x
29/01/2019	VBA 25D	VBA	M	Juvenil	27,99	3,33x10^0	79	+	-	x
29/01/2019	VBA 21D	VBA	M	Juvenil	27,44	4,94x10^0	77,5	+	-	x
29/01/2019	VBA 50	VBA	M	Filhote	27	6,72x10^0	77,5	+	-	x
30/01/2019	VBA 51	VBA	M	Juvenil	19,46	1,41x10^3	77,5	+	+	x
30/01/2019	VBA 52	VBA	F	Juvenil	24,41	4,27 x10^1	79,5	+	+	x
20/03/2019	VBA 03F	VBA	F	Adulto	24,12	5,39 x10^1	79,5	-	+	x
21/03/2019	VBA 21E	VBA	M	Subadulto	28,26	2,85x10^0	77,5	+	-	x
21/03/2019	VBA 53	VBA	F	Adulto	26,13	2,38 x10^1	79,5	+	+	x
21/03/2019	VBA 44B	VBA	M	Adulto	21,96	2,39x10^2	77,5	-	+	x
21/03/2019	VBA 54	VBA	M	Subadulto	Negative	Negative	-	+	-	x
22/03/2019	VBA 55	VBA	M	Adulto	22,19	2,03x10^2	79	+	+	x
25/03/2019	VBA 38B	VBA	F	Subadulto	28,05	3,18x10^0	79	+	-	x
25/03/2019	VBA 56	VBA	F	Subadulto	28,46	2,90x10^0	79	+	-	x
25/03/2019	VBA 57	VBA	F	Adulto	26,32	1,31 x10^1	79	+	-	x
26/03/2019	VBA 16D	VBA	F	Adulto	23,26	1,10x10^2	77,5	+	+	x

27/03/2019	VBA 25E	VBA	M	Subadulto	16,98	$9{,}03x10^3$	79,5	+	+	x
28/03/2019	VBA 08C	VBA	F	Adulto	20,26	$8{,}98x10^2$	79,5	+	+	x
23/04/2019	VBA 58	VBA	M	Filhote	28,64	$2{,}55x10^0$	79,5	-	-	x
23/04/2019	VBA 21F	VBA	M	Subadulto	14,27	$5{,}98x10^4$	77,5	+	+	x
23/04/2019	VBA 59	VBA	M	Adulto	21,79	$3{,}08x10^2$	79,5	+	+	x
23/04/2019	VBA 19B	VBA	F	Adulto	24,27	$5{,}48\ x10^1$	79,5	+	+	x
25/04/2019	VBA 60	VBA	M	Subadulto	22,75	$1{,}58x10^2$	79,5	+	+	x
26/04/2019	VBA 01B	VBA	M	Adulto	26,25	$1{,}36\ x10^1$	79	+	-	x
30/04/2019	VBA 44C	VBA	M	Adulto	22,49	$1{,}88\ x10^2$	77,5	+	+	x
30/04/2019	VBA 55B	VBA	M	Adulto	22,35	$2{,}09\ x10^2$	79	+	+	x

[1] Parque Estadual do Prosa; [2] Vila da Base Aérea; M: Male; F: Female

Board 2: Identification of coatis sampled and recaptured in the two locations (PEP and VBA), field number, red blood cells count, hemoglobin and hematocrit parameters determinate using hematology analyzer device (POCH-iV 100, Syxmex®, Brazil).

Identification	Red blood cells (10_6/µL)	Hemoglobin (g/dL)	Hematocrit (%)
PEP 02	5.7	11.5	33.7
PEP 04	5.76	10.7	31.4
PEP 06	5.86	11.6	33.1
PEP 08	4.76	9.6	29.2
PEP 10	4.9	9.5	29.1
PEP 11	5.52	10.1	31.5
PEP 13	6.44	13.3	38.2
PEP 14	6.15	11.1	33.1
PEP 15	5.94	11	31.7
PEP 17	5.84	11.5	33
VBA 01	5.29	10	30.1
VBA 03	5.26	9.2	29.4

VBA 04	4.88	9	28.6
VBA 05	4.66	9.1	28.8
VBA 06	6.08	12.3	35
VBA 07	5.77	12.2	34.9
VBA 08	5.78	10.8	32.9
VBA 09	5.41	10.9	32.6
VBA 10	5.06	9.9	30.2
VBA 11	6.32	12.5	36.4
VBA 12	4.86	8.9	28.5
VBA 13	6.07	11.2	34.6
PEP 18	5.97	10.7	32.8
PEP 19	6.43	11.2	34.9
PEP 20	5.41	9.9	30.5
PEP 21	5.58	11.2	33.6
PEP 22	5.74	11.7	34.5
PEP 23	5.38	11.1	32.6
PEP 24	5.06	10	30.5
PEP 25	5.7	10.5	33.3
VBA 15	6.04	12	36.4
VBA 16	6.14	11.4	34.6
VBA 17	4.91	10.3	31.7
VBA 18	5.42	10.5	30.9
VBA 19	5.49	10.5	31.1
VBA 20	6.41	12.5	38
VBA 21	5.85	11.5	34.7
VBA 22	5.8	11.4	34.1
VBA 23	5.85	10.1	32.2
VBA 24	5.6	10.8	33.4
VBA 25	5.93	11.6	35.2
VBA 26	5.26	9	26.5

PEP 27	5.12	10.1	30.6
PEP 28	5.2	11.1	32.8
PEP 29	5.37	10.4	32
PEP 30	5.23	9.7	30.5
PEP 31	5.91	11.3	34.6
PEP 32	5.13	10.4	32.4
PEP 33	5.75	11.2	35.2
PEP 34	5.1	9.6	30.5
PEP 35	5.1	9.1	29.1
PEP 36	4.71	9.5	29
PEP 37	5.18	9.8	30.1
PEP 38	4.79	10	28.4
PEP 39	5.61	11.3	34
PEP 40	5.45	10.9	33.5
PEP 42	4.96	9.4	28.9
PEP 43	5.92	11.6	35.2
VBA 27	5.9	12.9	37.9
VBA 28	5.12	10.9	32.9
VBA 30	5.51	10.7	33
VBA 31	3.98	8.4	26.1
VBA 32	4.75	9.8	30.3
VBA 33	5.74	11.2	34.7
VBA 34	5.46	10.3	32.1
VBA 35	5.33	9.8	31.6
VBA 36	5.31	10.4	32.3
VBA 37	4.42	9	28.7
VBA 38	5.4	11.2	35
VBA 40	5.15	11.1	32.7
PEP 44	5.08	10.3	32.1
PEP 45	5.12	9.5	30.8

PEP 46	5.24	9.7	31.1
PEP 47	5.72	10	32.3
PEP 48	5.33	9.8	31.5
PEP 49	5.24	10.5	32
PEP 50	4.92	9.2	29.5
PEP 51	5.11	10	30.7
VBA 41	4.61	9.8	30.8
VBA 42	5.44	10.6	33.2
VBA 43	5.75	11.6	34.7
VBA 44	5.3	11	34.3
VBA 45	5.27	10.3	32.8
VBA 46	5.43	9.7	30.8
VBA 47	5.25	10.7	33.2
VBA 48	5.63	10.6	33.4
VBA 49	5.09	9.8	30
VBA 50	6.39	12.5	36.8
VBA 51	6.02	11.1	34.4
VBA 52	5.53	10.5	32.4
VBA 53	5.62	10.2	31.7
VBA 54	5.14	9.8	29.2
VBA 55	5.09	10	30.6
VBA 56	5.53	10.7	32.6
VBA 57	4.96	9.2	28.9
VBA 59	5.79	12.3	35.8
VBA 60	5.49	11	33.1

Capítulo 8* - Molecular detection of piroplasmids and *Rickettsia* spp. in coatis (*Nasua nasua*) and associated ticks from midwestern Brazil

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Abstract

Procyonids are reservoirs of many zoonotic infectious diseases, including tick-borne pathogens. The role of coatis (*Nasua nasua*) in the epidemiology of piroplasmids and *Rickettsia* has not been fully addressed in Brazil. To molecular study these agents in coatis and associated ticks, animals were sampled in two urban areas in Midwestern Brazil. Blood (n=163) and tick (n=248) DNA samples were screened by PCR assays targeting the 18S rRNA and *gltA* genes of piroplasmids and *Rickettsia* spp, respectively. Positive samples were further molecularly tested targeting *cox-1*, *cox-3*, *β-tubulin, cytB* and *hsp*70 (piroplasmid) and *ompA*, *ompB* and *htrA* 17-kDa (*Rickettsia* spp.) genes, sequenced and phylogenetic analyzed. All coatis' blood samples were negative for piroplasmids, whereas five pools of ticks (2%) were positive for two different sequences of *Babesia* spp.. The first from *Amblyomma sculptum* nymphs was close (i.e., ≥ 99% nucleotide identity) to a *Babesia* sp. previously found in capybaras (*Hydrochoerus hydrochaeris*); the second from *Amblyomma dubitatum* nymphs and *Amblyomma* spp. larvae was identical (100% nucleotide identity) to a *Babesia* sp. detected in opossums (*Didelphis albiventris*) and associated ticks. Four samples (0.8%) were positive by PCR to two different *Rickettsia* spp. sequences, being the first from *Amblyomma* sp. larva identical to *Rickettsia belli* and the second from *A. dubitatum* nymph identical to *Rickettsia* species from Spotted Fever Group (SFG). The detection of piroplasmids and SFG *Rickettsia* sp. highlights the importance of *Amblyomma* spp. in the maintenance of tick-borne agents in urban parks where humans, wild and domestic animals are living in sympatry.

Keywords: Zoonosis, tick borne pathogens, *Babesia* sp., SFG *Rickettsia* sp., *Amblyomma sculptum, Amblyomma dubitatum*

1. Introduction

Among wild carnivores, ring-tailed coatis (Procyonidae, *Nasua nasua*) are medium size mammals with a wide geographic distribution in South America that easily adapt to different environments. They can be found in large familiar groups in urbanized areas, favoring contact with domestic animals and humans, enabling exchange of ectoparasites and associated pathogens (Emmons and Helgen 2016). Indeed, they are considered important sources of zoonotic agents, including tick-borne pathogens (TBP) (Cleaveland et al., 2001; Otranto et al., 2015; André, 2018).

Piroplasmids of the genera *Theileria, Babesia*, *Rangelia* and *Cytauxzoon* are tick-borne protozoa diffused worldwide, in both domestic and wild animals (Yabsley and Shock, 2013; Jalovecka et al., 2018; 2019; Schnittger et al., 2022). According to their biological life cycles, piroplasmids may be perpetuated transestadially and/or transmitted transovarially, resulting in large numbers of infected ticks in areas where piroplasmids are endemic (Uilenberg 2006; Friedhoff 2018). Other bacterial tick-borne pathogens, such as *Rickettsia* spp., are prevalent in animal population in association with their tick vectors (Abdad et al., 2018; Bermúdez and Troyo 2018). Under the above circumstances, wildlife plays an important role in the maintenance of the biological life cycle of both tick species and transmitted pathogens, representing a blood feeding source for ticks and the main reservoirs and amplifiers for many tick-borne pathogens (Cleaveland et al., 2001; Otranto et al., 2015; André, 2018).

Raccoons (*Procyon lotor*) may be infected with up to four *Babesia* spp. (i.e., *Babesia microti*-like sp., *Babesia lotori,* and two putative novel *Babesia* spp. from *Babesia* sensu stricto and Western *Babesia* clades) in the USA and Canada (Goethert and Telford 2003; Birkenheuer et al., 2007; Hersh et al., 2012; Clark et al., 2012; Garret et al., 2019) and a *Babesia-microti* like in Japan (Jinnai et al., 2009). In the reports above, the role of raccoons as reservoirs of *Babesia* sp. was highlighted with emphasis on the potential zoonotic of *B. microti* (Kawabuchi et al., 2005; Jinnai et al., 2009; Clark et al., 2012). A *Babesia* sp., molecularly classified within the *Babesia* sensu stricto clade, was detected in *Nasua narica* in Costa Rica and in *Procyon cancrivorous* from Uruguay (Mehrkens et al., 2013; Thompson et al., 2018). In addition, *Theileria* sp. was morphologically identified in coatis sampled in the Pantanal wetland (state of Mato Grosso do Sul, Brazil) and molecularly characterized, using partial 18S rRNA, as belonging to the clade of *Theileria equi,* albeit closely related to a *Theileria* sp. detected in a domestic cat (de Sousa et al., 2018).

Procyonids play an important role in the epidemiology of rickettsial agents. For instance, serological evidence of exposure to *Rickettsia rickettsii*, *Rickettsia montana*, *Rickettsia parkeri* and *Rickettsia bellii* (369-C strain) was reported in raccoons from the USA (Castellaw et al., 2011; Rainwater et al., 2017; Tufts et al., 2021). Additionally, other *Rickettsia* species were molecularly identified in skin fragments (e.g. *Rickettsia monacensis*, *Rickettsia raoultii*, '*Candidatus* Rickettsia kotlanii-like', *Rickettsia helvetica* and *Rickettsia* sp. endosymbionts) from introduced raccoons in Germany and Poland (Hildebrand et al., 2022) and in spleen samples (i.e., *Rickettsia japonica*, *Rickettsia felis*, *Rickettsia heliongjiangensis/R. japonica*, *Rickettsia amblyommi*, *R. helvetica* and *Rickettsia* sp. strain Hj126) from introduced feral raccoons in Japan (Sashika et al., 2010; Baba et al., 2013). In Brazil, *Rickettsia amblyommatis* and *R. bellii* were detected in *Amblyomma coelebs* in *Amblyomma ovale*, respectively, collected from coatis in Iguaçu National Park (Paraná state) (Magalhães-Matos et al., 2022).

Due to the Public Health relevance of coatis as reservoirs of tick-borne pathogens and their potential role in the maintenance and dispersion of ticks, this study aimed to detect and characterize molecularly piroplasmids and *Rickettsia* sp. in coatis and associated ticks sampled in two urban areas from central-western Brazil.

2. Material and Methods

Study area and sampling

Between March 2018 and January 2019, coatis were sampled in two Cerrado urban areas, 'Parque Estadual do Prosa' conservation unity (PEP site 1) (S20º 27' 8'', W54º 33' 40'') and the residential area 'Vila da Base Aérea' (VBA site 2) (S27º 51' 51'', W54º 39' 51''), both located in Campo Grande city (Mato Grosso do Sul State, Midwestern Brazil). All capture procedures were previously described (Barreto et al., 2021; Perles et al., 2022). Animals were captured using metal traps (1 m x 0.4 m x 0.5 m) placed arbitrarily according to the possibility of human access and availability of shadow, covering most of the PEP and VBA areas. Captured animals were anesthetized with an association of Tiletamine hydrochloride and Zolazepam hydrochloride (Telazol, Zoetis® (Parsippany-Troy Hills, NJ, USA) (6 mg/kg, intramuscularly). After chemical restraint, animals were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue between the shoulder blades. Of the 163 blood samples, 47 were collected from different individuals in site 1 (27 females and 20 males; 7 infants, 2 subadults and 38 adults) and 56 in site 2 (31 females and 25 males; 10 infants, 8 subadults and 38 adults). Blood samples were collected from the femoral vein using tubes containing EDTA (Ethylenediamine tetraacetic

acid), and then placed into RNAse/DNAse free cryotubes and stored at -80°C until molecular analyses. Animals' bodies were inspected, and ticks collected were stored in 100% ethanol for morphological identification (Martins et al., 2010; Dantas-Torres et al., 2019) and further molecular analyses.

Molecular analysis

DNA was extracted from 200 µL of blood using Illustra Blood Mini Kit (GE Healthcare®, USA) and from tick larvae and nymphs (in pools up to 10 and 5 individuals, from the same host) and individual adult ticks, using Biopur Mini Spin Plus kit (Mobius®, Brazil). To evaluate the quality of the extracted DNA and avoid false negative results, blood DNA samples were submitted to a conventional PCR (PCR) targeting the mammal *gapdh* (Glyceraldehyde 3-phosphate dehydrogenase) (Birkenheuer et al., 2003) and ticks' DNA samples were subjected to a PCR targeting the 16S rRNA gene of ectoparasites (Black and Piesman 1994).

Blood and tick DNA samples positive at PCR for endogenous genes were screened for piroplasmids DNA using a nested(n) PCR assay targeting a small fragment (~ 800 bp) of the 18S rRNA gene as previously described (Jefferies et al., 2007). All positive samples in the referred nPCR assay were subjected to further molecular characterization using conventional PCR assays targeting six molecular markers, namely 18S rRNA nearly complete gene (1,500 bp; Tsuji et al., 2006; Greay et al., 2018), *cox*1 (~ 800 bp; Corduneanu et al., 2017), *hsp*70 (~ 700 bp; Soares et al., 2011), *β-tubulin* (600 bp; Zamoto et al., 2004), *cytB* (~ 1 kb; Barbosa et al., 2019), and *cox*3 (~ 600 bp; Barbosa et al., 2019). *Babesia vogeli* DNA (Jaboticabal strain) was used as a positive control in PCR assays.

Ticks' DNA samples positive at PCR assay for the 16S rRNA endogenous gene were screened for *Rickettsia* spp. DNA using a PCR assay targeting a small fragment (~ 401 bp) of the *gltA* gene, as previously described (Labruna et al., 2004a). All positive samples were subjected to further molecular characterization using PCR assays targeting three molecular markers, namely *ompA* (530 bp) (Regnery et al., 1991), *ompB* (862 bp) (Roux and Raoult, 2000) and *htrA* 17-kDa (440 bp) (Labruna et al., 2004b). *Rickettsia vini* was used as positive control. Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in all PCR assays.

The amplicons obtained in conventional PCR assays were subjected to 1% ethidium bromide-stained agarose gel electrophoresis and visualized in a UV transilluminator (ChemiDoc MP Imaging System, Bio Rad®).

Sequencing and phylogenetic analyses

Amplicons were sequenced by Sanger's method using ABI PRISM 3730 DNA Analyzer (Applied Biosystems) at Human Genome and Stem Cell Research Center, "Instituto de Biociências", University of São Paulo (USP), Brazil. The BLASTn program was used to analyze consensus sequences, to compare sequences from the GenBank international database (https://www.ncbi.nlm.nih.gov/genbank/). For phylogenetic analyses, the alignment was performed using MAFFT software (https://mafft.cbrc.jp/alignment/server/) and Bayesian analyses were performed using CIPRES gateway (Ronquist and Huelsenbeck 2003). The phylogenetic tree edition and rooting (outgroup) were performed using TreeGraph 2.0 beta software (Stöver and Muller 2010).

3. Results

Tick identification

Ticks collected (n=2,242) were identified as *Amblyomma* larvae (n=838), *Amblyomma sculptum* nymphs (n=1241), *Amblyomma dubitatum* nymphs (n=150), *A. sculptum* adults (i.e., 3 males and 5 females), and *Amblyomma ovale* adults (i.e., 2 males and 3 females), as reported in Perles et al. (2022).

Molecular detection and analysis of piroplasmids

All DNA extracted from 163 coatis' blood samples were positive at the PCR targeting the *gapdh* housekeeping gene. On the other hand, 248 from 265 tick DNA pools samples were positive at the PCR targeting 16S rRNA (9 *Amblyomma* sp. larvae pools, 5 *A. sculptum* nymphs and 3 *A. dubitatum* nymphs were negative, and then excluded from the other PCR assays).

All coatis' blood samples were negative for piroplasmids, whereas five (2%) pools of ticks (one *Amblyomma* spp. larvae, two *A. dubitatum* nymphs and two *A. sculptum* nymph pools) were positive at the nPCR for piroplasmids.

The two sequences obtained from *A. sculptum* nymph pools showed ≥ 99% nucleotide identity with *Babesia* sp. (EF222255, OP714347) previously detected in capybaras (*Hydrochoerus hydrochaeris*) in Rio Grande do Sul and Goiás state, Brazil (fragments sizes 577 and 1,126 bp, 100% query-coverage and E-value of 0.0). The sequences obtained from one *Amblyomma* sp. larvae pool and two *A. dubitatum* nymph pools were identical (100% of nucleotide identity) with *Babesia* sp. previously detected in opossums (*Didelphis albiventris*) and associated *A. dubitatum* from the same studied area (MW342734, MW290048-MW290052) as well as opossums from Brasília, Federal District

(MW290046-MW290057) (fragments sizes 532, 889 and 1018 bp, 100% query-coverage and E-value of 0.0).

Phylogenetic analyses clustered the sequences detected in *Amblyomma* sp. larvae pool and the two *A. dubitatum* nymph pools with *Babesia* spp. previously detected in opossums and associated ticks, whereas sequences detected in *A. sculptum* nymph pools were grouped in a clade with *Babesia* sp. previously detected in wild rodents with 100% of bootstrap in both clades (**Figure 1**).

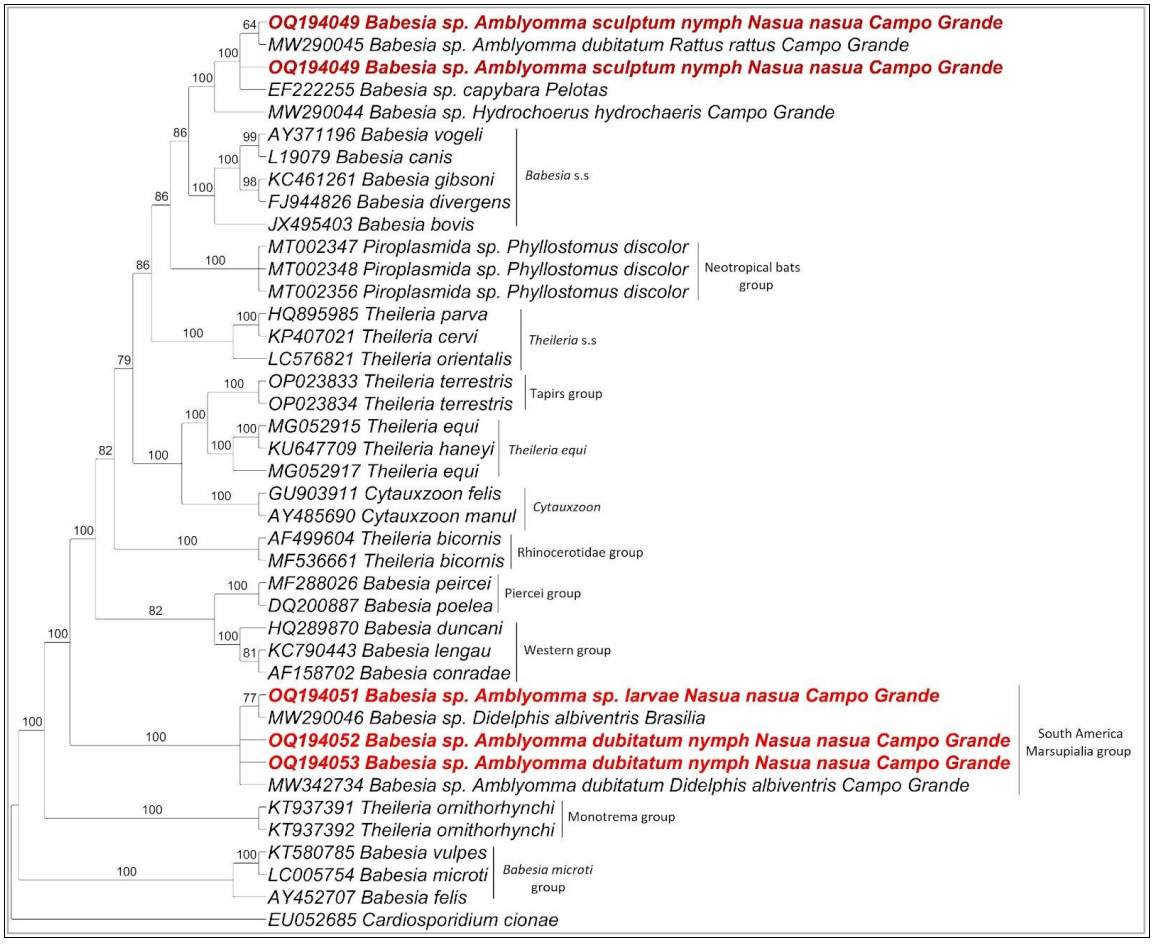

Figure 1. Phylogenetic tree based on Bayesian method of an alignment of 1,635 bp and TIM3+I+G4 evolutionary model of the 18SrRNA gene of piroplasmid detected in coati' ectoparasites samples from Campo Grande city, Mato Grosso do Sul state, Brazil. *Cardiosporium cionae* (EU052685) was used as outgroup. Numbers at the nodes correspond to posterior probabilities. Sequences of *Babesia* sp. detected in the present study were highlighted in red bold.

Even though two *A. dubitatum* nymphs' pools were positive at the cPCR targeting the *hsp-70* gene, the sequences obtained did not have a good quality and scored negative at the additional PCR assays targeting *cox1*, *β-tubulin*, *cytB,* and *cox3* genes.

Molecular detection and analysis of Rickettsia *spp.*

Four samples (0.8%), two *Amblyomma* sp. larvae pools and two *A. dubitatum* nymph pools, yielded positive at PCR for *Rickettsia* spp. Two *gltA* sequences obtained from *Amblyomma* sp. larvae were identical (100% nucleotide identity) to *R. belli* (fragment sizes 317 bp, 100% query coverage and 7e-163 E-value). Additionally, two *gltA* sequences obtained from *A. dubitatum* nymph pools were identical (100% nucleotide identity) with *Rickettsia* species from SFG (i.e., *R. sibirica*, *R. parkeri*, '*Candidatus* Rickettsia paranaensis') (fragment sizes 379 bp, 100% query coverage and 0.0 E-value). All four samples were negative at additional PCR assays targeting the *ompA*, *ompB,* and *htrA* 17-kDa genes. Phylogenetic analyses clustered the sequences detected in *Amblyomma* sp. larvae in a clade with *R. belli*, while the isolates detected in *A. dubitatum* nymphs grouped in a clade with several SFG *Rickettsia* species (**Figure 2**).

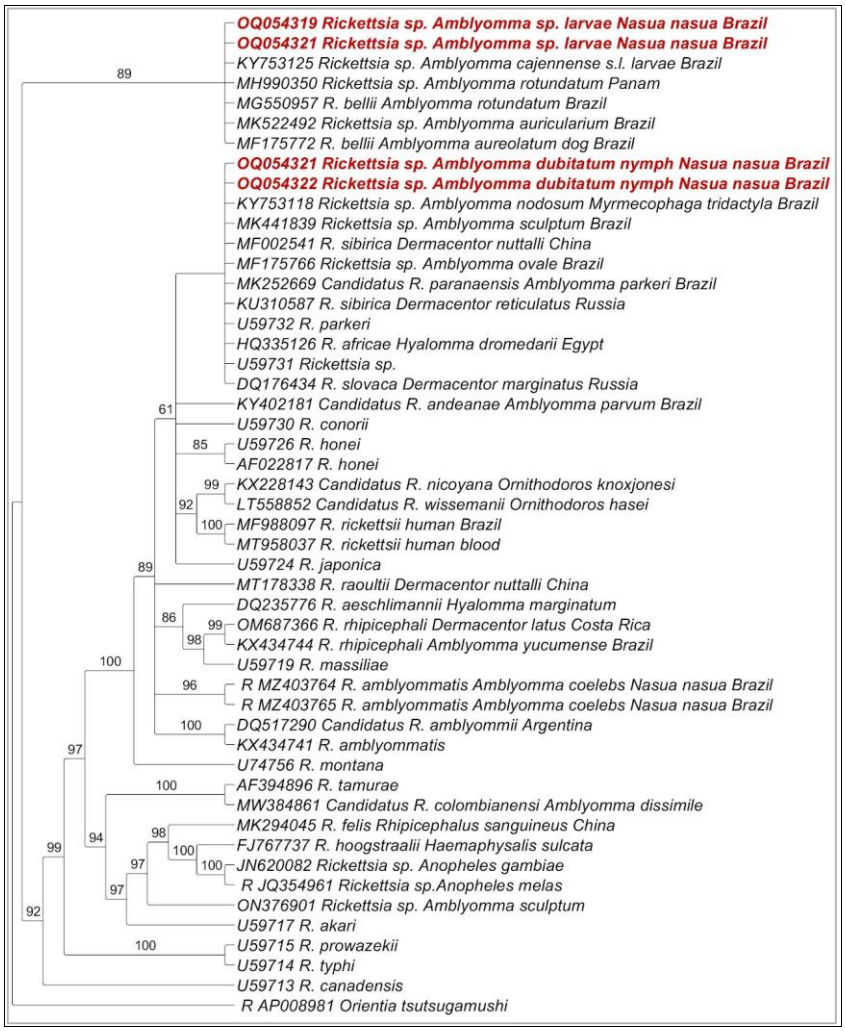

Figure 2. Phylogenetic tree based on Bayesian method of an alignment of 388 bp and GTR+G evolutionary model of the *gltA* gene of *Rickettsia* sp. detected in coati' ectoparasites samples from Campo Grande city, Mato Grosso do Sul state, Brazil. *Orientia tsutsugamushi* (AP008981) was used as outgroup. Numbers at the nodes correspond to posterior probabilities. Sequences detected in the present study were are highlighted in red bold.

4. Discussion

Data suggest that Piroplasmid and *Rickettsia* spp. are present in the coatis-associated ticks in urban parks from Campo Grande, representing a public health risk. In particular, the detection of a *Rickettsia* sp. from the SFG indicate that coatis are potential reservoirs of tick vectors, where they are living in close contact with humans and domestic animals. Similarly, procyonids were implicated in the dispersion of tick vectors of *R. rickettsii* and *B. microti*, in the USA (Clark et al., 2012; Tufts et al., 2021), Europe (Hildebrand et al. 2022) and Japan (Jinnai et al., 2009).

The detection of genomic DNA of *Babesia* spp. in associated ticks, but not in coatis' blood samples, requires further confirmatory studies about their susceptibility to both piroplasmids. One of the above *Babesia* sp. was previously found in opossum and in *A. dubitatum* associated-ticks from central-western Brazil and included in the "South American Marsupialia Group" (Gonçalves et al., 2021). Accordingly, the molecular identification of this *Babesia* sp. in *Amblyomma* larvae raises questions about the transovarial transmission of this agent. The other *Babesia* sp. from *A. sculptum* was phylogenetically clustered with *Babesia* sp. from *Amblyomma* sp. ticks collected in wild rodents from central-western Brazil (Gonçalves et al., 2021) and capybaras from the states of Rio Grande do Sul (Criado-Fornelio et al., 2009) and Goiás (Neves et al., 2023). Due to the hindrances in amplifying and sequencing piroplasmids in ticks collected from coatis and previously in capybaras (Gonçalves et al., 2021), future studies should better define the phylogeny of this complex group of piroplasmids, through the characterization of large fragments of 18S rRNA and mitochondrial genes. Similarly, experimental transmission studies should address the vectorial role of *A. sculptum* and *A. dubitatum*, found parasitizing capybaras in Brazil (Gonçalves et al., 2021; Neves et al., 2022).

In addition, coatis may play an important role in the dispersal of pathogenic *Rickettsia*-harboring ticks, since horses and capybaras have already been reported parasitized by *Rickettsia* sp. in the studied area (Campos et al., 2021; 2022). The central role of *A. sculptum* as vector in the transmission of SFG *Rickettsia* spp. in Brazil is already known (Polo et al., 2017). The evidence of transmission of the agent by the co-feeding of *A. sculptum* with *A. dubitatum* (Pacheco et al., 2007, Souza et al., 2009; Zemtsova et al., 2010; Moraes-Filho et al., 2018), and the confirmation of the maintenance of the rickettsiae trans-stadially by *A. dubitatum* (Sakai et al., 2014), suggest a role of the last species into the epidemiology of these infections. The detection of *R. bellii* in pool of *Amblyomma* sp. larvae from coatis confirms previous records of the same *Rickettsia* sp. in *Amblyomma* sp. ticks collected from other hosts, in Latin America (Labruna et al., 2007; Labruna et al., 2011; Barbieri et al., 2012). This species of *Rickettsia* is the most primitive one in the genus, but its pathogenicity remains unknown (Labruna et al., 2004b; McIntosh et al., 2015). However, numerous tick-borne rickettsiae that were initially considered without medical importance, are now recognized as causing diseases in humans (e.g., *Rickettsia parkeri* in the USA), showing the importance of pathogen surveillance in ticks (Parola et al., 2013).

Conclusions

Coatis from urban areas in Campo Grande city may harbor *A. dubitatum* and *A. sculptum* ticks infected with *Babesia* spp. in opossum and capybara, respectively. The role of

coatis in the perpetuation of the biological life cycle of ticks implicated in the transmission of SFG *Rickettsia* needs to be further investigated under experimental and field conditions.

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Statements and declarations

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Competing Interests
The authors declare no competing interests.

Availability of Data

The datasets generated and analysed during the current study are available in the NCBI GenBank Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession numbers: *Rickettsia* sp. *gltA* (OQ054319, OQ054320, OQ054321, OQ054322), *Babesia* sp. 18SrRNA (OQ194049 - OQ194053).

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Ethical Statement

All methods were carried out in accordance with relevant guidelines and regulations and were approved by the “Instituto Chico Mendes de Biodiversidade” (ICMBio) (SISBIO 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB 001/2018) and ‘Sistema Nacional de Gestão de Patrimônio Genético’ (ABDF0B5).

Consent to participate

Not applicable

Consent to publish

Not applicable

Capítulo 9* – *Mansonella* sp. and associated *Wolbachia* endosymbionts in ring-tailed coatis (*Nasua nasua*) in periurban areas from midwerstern Brazil

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Abstract

Coatis (*Nasua nasua*) are wild carnivorous well adapted to anthropized environments especially important because they act as reservoirs hosts for many arthropod-borne zoonotic pathogens. Information about filarioids from coatis and associated *Wolbachia* spp. in Brazil is scant. To investigate the diversity of filarial nematodes, blood samples (n= 100 animals) were obtained from two urban areas in midwestern Brazil and analyzed using morphological by blood smear and buffy coat and molecular tools using cPCR assays based on *cox*1, 12S rRNA, 18S rRNA, *hsp*70 and *myo*HC genes for nematodes and 16S rRNA for *Wolbachia*. When analyzing coati blood smears and buffy coats, 33% and 80% of the samples presented at least one microfilaria, respectively. Twenty-five *cox*1 sequences were obtained showing 89% nucleotide identity with *Mansonella ozzardi*. Phylogenetic analyses clustered *cox*1 sequences herein obtained within the *Mansonella* spp. clade. Sequences of both *myo*HC and two *hsp*70 genes showed 99.8% nucleotide identity with *Mansonella* sp. and clustered into a clade within *Mansonella* sp., previously detected in coatis from Brazil. Two blood samples were positive for *Wolbachia*, with a 99% nucleotide identity with *Wolbachia* previously found in *Mansonella perstans, Mansonella ozzardi* and *Mansonella atelensis* and in ectoparasites of the genus *Pseudolynchia, Melophagus* and *Cimex*. The study showed a high prevalence of *Mansonella* sp. in the coati population examined, suggesting that this animal species play a role as reservoirs of a novel, yet to be described, species within the Onchocercidae family.

Key-Words: Onchocercidae, *Mansonella*, *Wolbachia*, Procyonidae, endosymbiont

Introduction

Filarioids belonging to the Onchocercidae family can be transmitted by a plethora of arthropod vectors (e.g., mosquitoes, black flies, midges, ticks, lice and mites) to several animal species, including humans [1, 2, 3, 4, 5, 6, 7, 8]. Some of these agents have a zoonotic potential, such as *Dirofilaria immitis,* which causes heartworm disease in dogs, and *Dirofilaria repens,* which may cause a non-pathogenic subcutaneous infection in humans [8, 9]. While the former is the main causative agent of human dirofilariasis in the United States, the latter is more prevalent in the Old World [10].

The biology and epidemiology of filarioids has been extensively investigated in domestic animals [4, 11] and, at a lesser extent, in wildlife, especially in geographical areas with a huge host biodiversity [12, 13]. It is the case of Brazil, where a novel *Dirofilaria* species was extracted from the eye of a young patient in the Amazon forest [14]. Wild canids (e.g., jackals, foxes, wolves) have been incriminated as reservoirs for *D. immitis* in different regions of the world [15], contributing to the endemicity of such agent where domestic animals are under chemoprophylactic treatment [7]. Furthermore, in the United States, *Dirofilaria tenuis* is a common parasite of raccoons (*Procyon lotor*), that has also been detected in a wrist lesion of a healthy young woman [16].

In filarial nematodes, *Wolbachia* endosymbionts are intracellular obligatory bacteria involved in their reproduction, development, and long-term survival [17, 18, 19]. Accordingly, *Wolbachia* are vertically transmitted to the progeny through the eggs cytoplasm, being mainly associated to female nematodes reproductive system [20, 21]. These endosymbionts are classified in supergroups, based on their genetic evolution in a large variety of hosts [19, 22], with those found in nematodes belonging to the supergroups C, D [22] and F [23, 24, 25, 26].

Ring-tailed coatis (*Nasua nasua*) are medium-sized Procyonids widely distributed in South America (e.g., Brazil, Paraguay) that easily adapt from forestry to urbanized ecosystems [27, 28] frequently resulting in parasite exchanges with other

animal species). For example, at least three Onchocercids have been simultaneously detected in coatis from Paraná State, southern Brazil, suggesting their role as reservoirs of zoonotic filarioids [29, 30]. Nonetheless, microfilariae from coatis could not be identified at species level, due the paucity of reference heat shock protein (*hps*70) and myosin heavy chain (*myo*HC) sequences available in genetic repertoires [29, 30]. Conversely, many filarioids have been barcoded by using a dual approach that combines morphological key characters for adult helminths and sequencing of three molecular markers, namely 12S rRNA, cytochrome *c* oxidase subunit 1 (*cox*1) and 18S rRNA [2].

Overall, procyonids are receptive hosts for filarioids since, for example, raccoons were found infected with *D. immitis*, *D. tenuis, Acanthocheilonema procyonis, M. llewellyni, Brugia beaveri* and *Dracunculus insignis*, in the USA [31, 32, 33, 34, 35, 36] and coatis with *Dirofilaria incrassata*, *Dirofilaria repens* and *Dirofilaria* sp. [37] and *Dirofilaria* sp., *Mansonella* sp. and one Onchocercidae yet to be characterized [29, 30], in Brazil. Therefore, the diversity in Onchocercidae species in Procyonidae underpins the role played by these carnivorous as reservoirs for zoonotic filarioids. Given the paucity of information about the life history of filarioids parasitizing coatis from Brazil, and the possibility that they arbor species of importance for health of human and their domestic animals, we characterized morphologically and molecularly blood microfilariae and assessed the prevalence of infection in coatis sampled in two urban fragments from Campo Grande city, Brazil. Also, data were corroborated by molecular information of *Wolbachia* endosymbionts.

Material and Methods

Blood sampling and microfilariae examination

Between March of 2018 and January of 2019, coatis were sampled every three months during 10 consecutive nights in two sampling spots [*Parque Estadual do Prosa* (PEP) (-20.44987, -54.56529)] and Brazilian Air Force Private Area (VBA) (-20.47163, -54.65405)], in Cerrado biome, located in Campo Grande city, Mato Grosso do Sul State, Midwestern Brazil. All captures were performed as previously

described [38, 39]. Briefly, blood samples obtained from femoral vein were stored in tubes containing EDTA (Ethylenediamine tetraacetic acid) and divided for two analyses: a) microhematocrit to obtain buffy coat and blood smears stained with Diff Quick for visual detection of microfilaria b) DNA extraction for molecular analyses. The morphological examination of the microfilariae was performed using an optical microscopy (Leica DM-LB2) by measuring according to the microfilariae available (i.e., from 1 to 5 specimens for each sample) according scientific literature [40] with Leica Las version 4.5.0 software (Leica Microsystems, Wetzlar, Germany) after Diff Quick stain.

Molecular assays

DNA was extracted from 200 µL of each coati blood sample using Illustra Blood Mini Kit (GE Healthcare®, USA), according to the manufacturer's instructions. To evaluate the quality of the extracted DNA and avoid false negative results, DNA samples were tested by a conventional PCR targeting the mammal *gapdh* [41]. Twenty-five samples presenting microfilaria at buffy coat analysis were randomly chosen randomly and were submitted to a PCR assay using filarioid-generic primers, namely NTF (5′-TGATTGGTGGTTTTGGTAA-3′) and NTR (5′-ATAAGTACGAGTATCAATATC-3′), which amplify a portion (689 bp) of the mitochondrial *cox*1 gene [42]. For further characterization, samples were submitted to PCR assays targeting the 12S rRNA, 18S rRNA [43, 44, 45], *hsp*70*,* and *myoHC* genes [30]. Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in all PCR assays. *Thelazia callipaeda* DNA [46] was used as a positive control.

The presence of *Wolbachia* DNA was tested through a PCR targeting the 16S rRNA gene [47] in the same samples submitted to cPCR screening for filarioids. PCR products were subjected to 2% gel red-stained agarose gel electrophoresis and results were visualized in UV transilluminator. Amplicons were purified and sequenced in both directions using the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic Analyzer (Applied Biosystems, California, USA) in an automated sequencer (ABI-PRISM 377).

Bioinformatic analyses

Sequences obtained were compared with those available in GenBank database through BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [48]. For phylogenetic inferences, sequences from the present study were aligned with those retrieved from GenBank using MAFFT software version 7 [49]. The best evolutionary model was chosen under the Akaike Information Criterion (AIC) and Maximum likelihood (ML) phylogenetic analyses was performed using the iqTREE software (available at: http://iqtree.cibiv.univie.ac.at/). The phylogenetic tree edition and rooting (outgroup) were performed using TreeGraph 2.0 beta software [50]. Protein translation for *cox1* gene was performed with MEGA software [51]. Interspecific and intraspecific nucleotide divergence and the presence of haplotypes were evaluated according criteria previously established by Ferri et al. [45].

Results

Of the 100 coatis captured (n=42 in PEP and n=58 in VBA; 57 females, 43 males) 30% and 80% scored positive for microfilariae at blood smears and buffy coat, respectively.

The microfilariae were serpentine in shape had no sheath and measured 190.5 ± 14.4 µm in length and 3.29 ± 0.28 µm width. Anterior end was rounded with short head space and presented cephalic space with scattered fine nuclei (**Figure 1**). The tail was long, slender, pointed with nuclei to the end. Body appeared with compact column of the nuclei purple in color, extending to the end of the tail (**Figure 1**). The nuclei were sparse osmogenes in the region of the inner body with exception of two big round areas without nuclei, in the initial third of the body (about 50-55 µm from anterior end) and in the last third, before the tail (about 30-35 µm from anterior end) (**Figure 1**). Based on the above morphological character's specimens were morphologically identified belonging to the genus *Mansonella*.

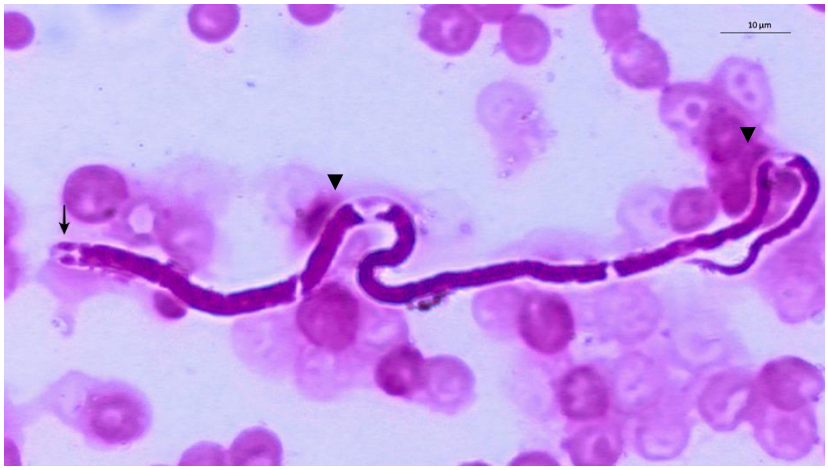

Figure 1. Microfilaria of *Mansonella* sp. from coati (*Nasua nasua*) blood smear stained with Diffy Quicky. Body of the microfilariae with compact column of the nuclei purple in color, sparse osmogenes in the region of the inner body with exception of two big round areas without nuclei (arrow head), with anterior end rounded with short head space and cephalic space with scattered fine nuclei (arrow).

Blast of 25 *cox*1 sequences (603 bp) showed 88-89% nucleotide identity (100% of query coverage and 0.0 E- value) *M. ozzardi* from human (MK491767). In addition, *cox*1 sequences revealed two different haplotypes with 15 silent mutations (**Table 1).**

Table 1. Nucleotide mutations of two *cox*-1 haplotypes of *Mansonella* sp. detected in blood samples from coatis (*Nasua nasua*) from Campo Grande city, Mato Grosso do Sul state, Brazil.

Nucleotide position	**28**	**91**	**148**	**160**	**193**	**205**	**206**	**211**	**315**	**388**	**436**	**446**	**451**	**547**	**577**
Haplotype 1	C	G	C	C	C	T	T	T	A	A	A	C	T	C	G
Haplotype 2	T	A	T	T	T	C	C	C	G	G	G	T	C	T	A

Two *hsp*70 sequences (468 pb) showed 99.7% nucleotide identity (100% of Query Coverage and 0.0 of E- value) with *Mansonella* sp. (MW330348-49) previously detected in coatis from Paraná state, southern Brazil. The only *myo*HC sequence showed 99.8% identity (99% of Query Coverage and 0.0 of E- value) with

Mansonella sp. previously detected in coatis from Paraná state (MW330367). All nucleotide sequences of *cox*1 and of *hsp70* and *myoHC* were clustered in a large *Mansonella* spp. clade (**Figure 2A, B, C**), with those of the latter genes grouped along with a *Mansonella* sp. previously detected in coatis from Brazil **(Figure 2B, C**). No sequences were obtained for 12S rRNA and 18S rRNA genes.

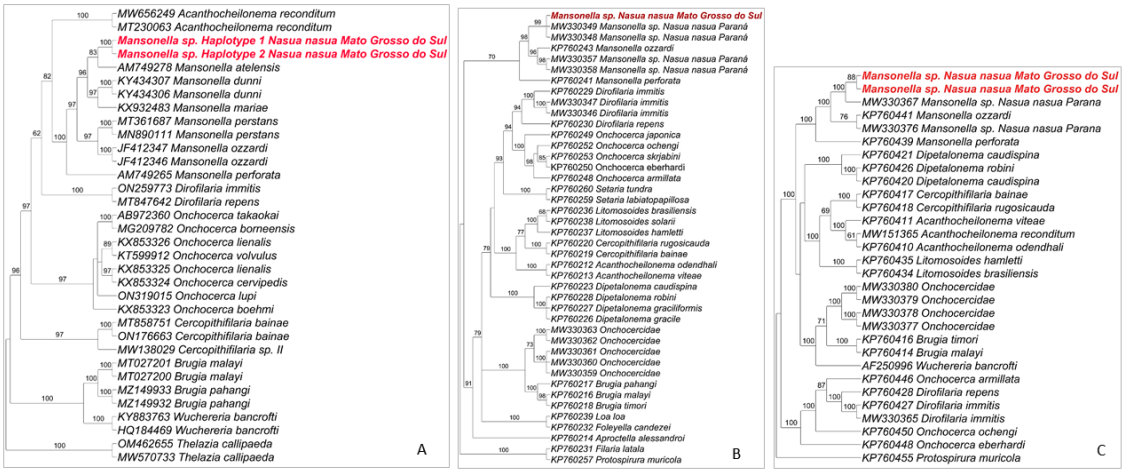

Figure 2. Phylogenetic tree based on Maximum likelihood inferences from filarioids detected in blood samples from coatis (*Nasua nasua*) from Campo Grande city, Mato Grosso do Sul state, Brazil. **A)** *cox*1 gene, *Thelazia callipaeda* was used as outgroup (alignment of 681 bp and TIM3+G as evolutionary model); **B)** *myoHC* gene, *Filaria latala* and *Protospirura muricola* were used as outgroup (alignment of 480 pb and HKY+G as evolutionary model); **C)** *hsp70* gene, *Protospirura muricola* was used as outgroup (alignment of 545 pb and TN+G as evolutionary model).

The two *Wolbachia* 16S rRNA sequences obtained showed >99% nucleotide identity (100% of query coverage and 1e-171 of E- value) with *Wolbachia* spp. previously detected in *M. perstans* (AY279355), *M. ozzardi* (AJ279034) *M. atelensis* (FR827940) and in *Pseudolynchia* sp. (MF461471), *Melophagus ovinus* (KY224164), *Cimex lectularis* (KU255228). Maximum likelihood phylogenetic analyses clustered the two *Wolbachia* sequences closely related to *Wolbachia* spp. previously detected in *Mansonella* spp. (Supergroup F) (**Figure 3**).

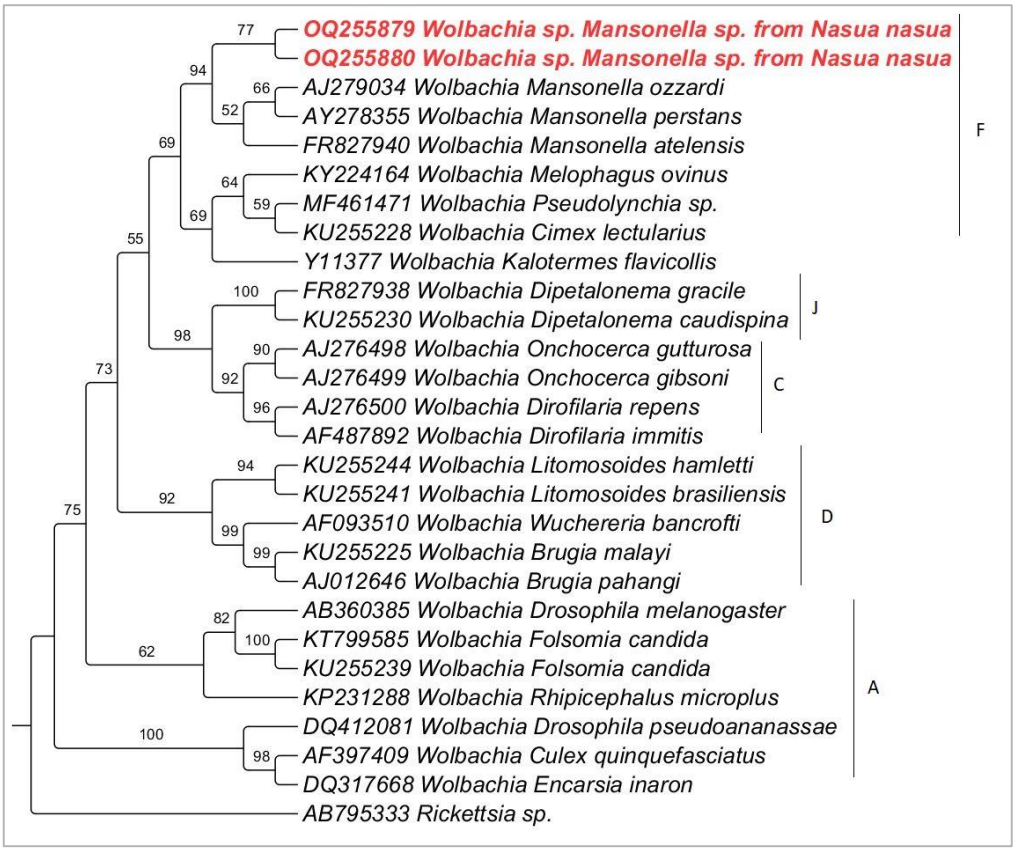

Figure 3. Phylogenetic tree based on Maximum likelihood inferences from 16SrRNA of *Wolbachia* sp. detected in blood samples from coatis (*Nasua nasua*) from Campo Grande city, Mato Grosso do Sul state, Brazil. *Rickettsia* sp. was used as outgroup. Analyses were performed based on an alignment of 337 bp and K2P+G4 as evolutionary model.

Discussion

Coatis herein examined were infected by *Mansonella* sp. blood circulating microfilariae suggesting that this animal species is a host for this onchocercid of potential zoonotic interest. Indeed, *Mansonella* includes a variety of species infecting a large number of different mammal hosts, such as carnivores, ungulates, sciurids, and primates [52]. Of those, *M. perstans*, *M. ozzardi* and *M. streptocerca* have a zoonotic potential [53], being reported in South and Central America (i.e., *M. perstans* and *M. ozzardi*) [53, 54] and in Africa (i.e., *M. perstans* and *M. streptocerca*) [53, 55].

Though morphological and molecular information prevent a proper diagnosis at species level, microfilarie were morphologically identified as *Mansonella* sp. The only species from the genus found in Procyonidae is *Mansonella llewellyni*, first described in subcutaneous tissue of raccoon in Maryland, USA [56]. This species was also found in different regions of USA in raccoons [36, 58] with microfilariae differing from those found herein in coatis, being 290 µm in length, with long tail ending in delicate button-hook curve without nuclei [56]. Furthermore, molecular evidence confirmed the identification at genus level as *Mansonella* sp. clustering sequences obtained with *M. ozzardi* and *M. perstans*, both of zoonotic concern. Even though the biological life cycle of this filariod from coatis is not known, they could be transmitted by Ceratopogonidae and Simuliidae, being the latter vectors for *Mansonella* genus [1]. The presence of coatis in urban areas may favor pathogens transmission exchange to humans and domestic animals.

Data suggest that the use of two or more different molecular markers is necessary for an accurate taxonomical identification of filarioids [2]. Herein we provide the first sequencing of *cox*1 gene from Procyonids filarioids, the most used molecular marker for molecular taxonomy [2]. Regarding the *hsp*70 and *myo*HC genes used as markers, the low genetic divergence detected supports the identification as the *Mansonella* species found in coatis from Paraná state [30]. Future studies are necessary to morphologically identify this putative novel *Mansonella* species through the description of adult nematodes and molecular confirmation.

Conclusions

Coatis may play a role as reservoirs of a novel yet to be described *Mansonella* species, that might show a potential zoonotic risk. Considering the significance of the genus, further studies are needed to identify its pathogenicity and to elucidate its biological life cycle.

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Ethical statement

All experimental procedures were approved by the “Instituto Chico Mendes de Biodiversidade” (ICMBio) (SISBIO 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB 001/2018) and Air Force Cooperation Agreement (Nº01/GAP-CG/2018).

Data availability statement

The datasets generated and analysed during the current study are available in the NCBI – GenBank – Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession numbers: *Wolbachia* sp. 16SrRNA (OQ255879-OQ25588); *Mansonella* sp. *cox1* (OQ261670-OQ261693); *hsp7*0 ( ); *myoHC* ( ).

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Declaration of interest

The authors declare that they have no conflict of interest.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by L.P., G.C.M. and W.T.G.B. The first draft of the manuscript was written by all authors. Funding acquisition was performed by Marcos Rogério André. All authors read and approved the final manuscript.

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Capítulo 10 - Considerações Finais

O presente trabalho teve como objetivo investigar a ocorrência molecular e diversidade de agentes Anaplasmataceae, Bartonellaceae, Mycoplasmataceae, Babesiidae/Theileriidae, Hepatozoidae e Onchocercidae, além da presença de filarídeos em esfregaços sanguíneos, de quatis amostrados em duas regiões urbanas da cidade de Campo Grande, estado do Mato Grosso do Sul, Brasil. A relação dos animais positivos aos hemoparasitas testados no presente estudo, com indicação das co-infecções está presente no quadro 1. Adicionalmente, o presente trabalho objetivou descrever os ectoparasitos presentes nos quatis amostrados.

Todos os animais analisados foram negativos para os ensaios moleculares para Piroplasmídeos) e microbiológicos e moleculares para *Bartonella* spp.. Dois animais (2/163 – 1,2%) foram negativos para todos os hemoparasitas pesquisados. Trinta e uma (31/163 – 19%) amostras de sangue de quatis foram positivas para somente um hemoparasita, sendo:

- Quatro amostras para filarídeos;

- Quatro amostras para *H. procyonis*;

- 23 amostras para hemoplasmas, dentre as quais 18 para o genótipo 1 e 4 para o genótipo 2.

Setenta e uma amostras de sangue de quatis (71/163 – 43,5%) apresentaram coinfecção por dois hemoparasitas, sendo:

- Uma amostra para *Neorickettsia* sp. e hemoplasma (genótipo 1);

- Duas amostras para *H. procyonis* e '*Candidatus* Ehrlichia dumleri';

- Duas amostras para *Anaplasma* sp. e hemoplasma (genótipo 1);

- Quatro amostras para *H. procyonis* e filarídeos;

- Quatro amostras para '*Candidatus* Ehrlichia dumleri' e hemoplasma (genótipo 1);

- 14 amostras para filarídeos e hemoplasma (genótipo 1);

- 44 amostras para *H. procyonis* e hemoplasma (genótipo 1),

Cinquenta e duas amostras de sangue de quatis (52/163 – 32%) foram positivas para três hemoparasitas, sendo eles:

- Uma amostra para *H. procyonis*, filarídeos e '*Candidatus* Ehrlichia dumleri';

- Uma amostra para *Neorickettsia* sp., '*Candidatus* Ehrlichia dumleri' e hemoplasma (genótipo 1);

- Uma amostra para filária, *Neorickettsia* sp. e hemoplasma (genótipo 2);

- Uma amostra para filária, *Anaplasma* sp. e hemoplasma (genótipo 1);

- Duas amostras para *H. procyonis*, *Anaplasma* sp. e hemoplasma (genótipo 2);

- Seis amostras para filárias, '*Candidatus* Ehrlichia dumleri' e hemoplasma (genótipo 1);

- Seis amostras para *H. procyonis*, '*Candidatus* Ehrlichia dumleri' e 'hemoplasma (genótipo 1);

- 34 amostras para *H. procyonis*, filarídeos e hemoplasma (genótipo 1);

Seis amostras de sangue de quatis (6/163 – 3,7%) foram positivas para quatro hemoparasitas, sendo:

- Uma amostra para filarídeos, *Neorickettsia* sp., *Anaplasma* sp. e hemoplasma (genótipo 1);

- Uma amostra para *H. procyonis*, filarídeo, *Neorickettsia* sp. e hemoplasma (genótipo 2);

- Quatro amostras para *H. procyonis*, '*Candidatus* Ehrlichia dumleri' e hemoplasma (genótipo 1).

Somente um quati foi positivo para cinco hemoparasitas, sendo eles *H. procyonis*, filarídeos, *Neorickettsia* sp., '*Candidatus* Ehrlichia dumleri' e hemoplasma (genótipo 1).

O presente trabalho corrobora estudos anteriores no que diz respeito à ocorrência de pelo menos dois genótipos de hemoplasmas circulantes em quatis. O presente trabalho mostra a co-infecção por diversos hemoparasitas (bactérias, protozoários e nematódeos) em quatis. Foi observado um alto índice de co-infecção, sendo que a infecção por *H. procyonis* e hemoplasmas mostrou-se como a mais frequente. As recapturas realizadas permitiram a observação das alterações hematológicas causadas pelos por *H. procyonis*, demonstrando uma diminuição de leucócitos com o aumento da parasitemia por este protozoário. Em relação aos agentes Anaplasmataceae, o presente trabalho relata, pela segunda vez, a circulação de *Neorickettsia* sp. em quatis no Brasil. Porém, devido a limitações metodológicas, não foi possível realizar a caracterização da bactéria. Por outro lado, foi possível realizar a caracterização molecular de um novo agente, descrito como '*Candidatus* Ehrlichia dumleri', em homenagem ao Prof. Dr. John Stephen Dumler. O presente estudo corrobora estudos anteriores conduzidos no estado do Paraná acerca da ocorrência de

Mansonella spp. em quatis no Brasil. Ectoparasitas foram coletados dos animais amostrados. Interessantemente, foi possível realizar o segundo relato de parasitismo por *A. dubitatum* em quatis. Com as recapturas e coletas em diferentes períodos do ano, foi possível realizar o estudo observacional da presença dos diferentes estádios (larvas, ninfas e adultos) de carrapatos ao longo do ano. Também foi possível realizar uma melhor descrição da espécie de piolho amostrada, *Neotrichodectes (Nasuicola) pallidus*. Esta espécie de piolho já foi descrita parasitando animais da família Procyonidae em outras regiões do Brasil, porém, o presente estudo permitiu a descrição molecular e realizou a primeira coleta de estágios de ovos da espécie, permitindo uma contribuição taxonômica. Quatis na cidade de Campo Grande carreiam carrapatos infectados com babesídeos parasitas de gambás e capivaras, além de uma *Rickettsia* sp. do Grupo da Febre Maculosa a ser caracterizada.

Quadro 1. Identificação dos quatis (*Nasua nasua*) amostrados em duas áreas urbanas da cidade de Campo Grande, Mato Grosso do Sul, com indicação do sexo e faixa etária dos animais amostrados e positividade aos hemoparasitas pesquisados.

N° de campo	Localidade	Sexo	Faixa etária	*Hepatozoon procyonis*	Filária	*Neorickettsia* sp.	*Ehrlichia* sp.	*Anaplasma* sp.	*Bartonella* sp.	*Mycoplasma* sp.	Piroplasmídeos
PEP 02	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	6,97x102	Negativo
PEP 04	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
PEP 06	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,84x102	Negativo
PEP 08	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,62x102	Negativo
PEP 10	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,57x101	Negativo
PEP 11	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,03x102	Negativo
PEP 13	PEP	M	Subadulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	5,27x103	Negativo
PEP 14	PEP	M	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	3,45x101	Negativo
PEP 15	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	4,21x101	Negativo
PEP 17	PEP	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	2,86 x101	Negativo
VBA 01	VBA	M	Adulto	Negativo	Positivo	Negativo	Positivo	Negativo	Negativo	2,32x102	Negativo
VBA 03	VBA	F	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	5,02 x101	Negativo
VBA 04	VBA	F	Adulto	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	5,75x101	Negativo
VBA 05	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	4,00x102	Negativo
VBA 06	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,62x102	Negativo
VBA 07	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	3,18x102	Negativo
VBA 08	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	2,25x102	Negativo
VBA 09	VBA	M	Subadulto	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo
VBA 10	VBA	F	Adulto	Negativo	Positivo	Positivo	Negativo	Negativo	Negativo	1,05x103	Negativo
VBA 11	VBA	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 12	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	9,60x100	Negativo
VBA 13	VBA	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	8,10x102	Negativo
PEP 18	PEP	F	Filhote	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
PEP 19	PEP	F	Filhote	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	5,36 x101	Negativo
PEP 20	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,97x102	Negativo
PEP 21	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	4,82 x101	Negativo
PEP 22	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,87x102	Negativo

PEP 02B	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	8,76x102	Negativo
PEP 01B	PEP	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	2,02x104	Negativo
PEP 03B	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,41x102	Negativo
PEP 05B	PEP	M	Adulto	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	5,28x101	Negativo
PEP 23	PEP	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,89x102	Negativo
PEP 12B	PEP	F	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	1,65x102	Negativo
PEP 24	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Positivo	Negativo	1,12x102	Negativo
PEP 25	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,11x104	Negativo
PEP 04B	PEP	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	6,83x102	Negativo
PEP 17B	PEP	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,83x102	Negativo
VBA 03B	VBA	F	Adulto	Positivo	Positivo	Positivo	Positivo	Negativo	Negativo	3,95x102	Negativo
VBA 15	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	5,27 x103	Negativo
VBA 12B	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	7,47x100	Negativo
VBA 08B	VBA	F	Adulto	Negativo	Negativo	Positivo	Negativo	Negativo	Negativo	5,49 x101	Negativo
VBA 10B	VBA	F	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	6,79 x101	Negativo
VBA 16	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	5,16x102	Negativo
VBA 17	VBA	F	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	8,10 x103	Negativo
VBA 11B	VBA	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,88x104	Negativo
VBA 18	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	1,30 x103	Negativo
VBA 19	VBA	F	Adulto	Negativo	Negativo	Positivo	Positivo	Negativo	Negativo	2,51x102	Negativo
VBA 20	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	5,61 x101	Negativo
VBA 21	VBA	M	Filhote	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,59x100	Negativo
VBA 07B	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	3,52x102	Negativo
VBA 22	VBA	F	Filhote	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	5,14x100	Negativo
VBA 23	VBA	F	Adulto	Negativo	Negativo	Negativo	Positivo	Negativo	Negativo	7,45x100	Negativo
VBA 24	VBA	M	Subadulto	Positivo	Positivo	Positivo	Negativo	Negativo	Negativo	5,71x100	Negativo
VBA 09B	VBA	M	Subadulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 25	VBA	M	Filhote	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 26	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	5,51x100	Negativo
PEP 23B	PEP	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,36 x103	Negativo

PEP 04C	PEP	M	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	3,80 x103	Negativo
PEP 27	PEP	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	5,29x102	Negativo
PEP 28	PEP	M	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	1,90x102	Negativo
PEP 29	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,97x102	Negativo
PEP 30	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	2,16x102	Negativo
PEP 03C	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	NR	Negativo
PEP 20B	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,63x102	Negativo
PEP 31	PEP	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,38 x103	Negativo
PEP 05C	PEP	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	3,43x102	Negativo
PEP 24B	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,65x102	Negativo
PEP 32	PEP	M	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	1,61x103	Negativo
PEP 33	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	2,06x102	Negativo
PEP 34	PEP	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	3,68 x101	Negativo
PEP 35	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
PEP 36	PEP	F	Subadulto?	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,13 x101	Negativo
PEP 12C	PEP	F	Subadulto?	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	4,09x102	Negativo
PEP 37	PEP	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	1,26x104	Negativo
PEP 38	PEP	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	3,57x102	Negativo
PEP 39	PEP	M	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	3,04 x101	Negativo
PEP 40	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	6,38 x101	Negativo
PEP 42	PEP	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	5,55x102	Negativo
PEP 01C	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	2,66x102	Negativo
PEP 43	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	6,30x102	Negativo
PEP 26B	PEP	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	3,76x102	Negativo
VBA 10C	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	7,16 x101	Negativo
VBA 27	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 07C	VBA	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	2,90x102	Negativo
VBA 28	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	7,46x102	Negativo
VBA 16B	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,31 x101	Negativo
VBA 21B	VBA	M	Filhote	Negativo	Positivo	Negativo	Negativo	Positivo	Negativo	5,64x100	Negativo

VBA 29	VBA	M	Filhote	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	NR	Negativo
VBA 30	VBA	F	Filhote	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,68 x101	Negativo
VBA 31	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,08x102	Negativo
VBA 32	VBA	F	Adulto	Positivo	Positivo	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo
VBA 25B	VBA	M	Filhote	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 33	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	2,32 x101	Negativo
VBA 34	VBA	M	Subadulto	Positivo	Positivo	Negativo	Positivo	Negativo	Negativo	2,17x102	Negativo
VBA 35	VBA	M	Subadulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	2,21 x101	Negativo
VBA 36	VBA	M	Filhote	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	2,62 x101	Negativo
VBA 03C	VBA	F	Adulto	Positivo	Positivo	Negativo	Positivo	Negativo	Negativo	2,12x102	Negativo
VBA 37	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	2,08x102	Negativo
VBA 38	VBA	F	Filhote	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	6,33x100	Negativo
VBA 22B	VBA	F	Filhote	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	5,39x100	Negativo
VBA 09C	VBA	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 40	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,03x102	Negativo
PEP 18B	PEP	F	Filhote	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
PEP 44	PEP	M	Filhote	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	7,88x100	Negativo
PEP 45	PEP	M	Filhote	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	9,85x102	Negativo
PEP 46	PEP	F	Filhote	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	3,93x100	Negativo
PEP 47	PEP	M	Filhote	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	7,78x100	Negativo
PEP 31B	PEP	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	4,66x103	Negativo
PEP 48	PEP	F	Adulto	Negativo	Negativo	Negativo	Negativo	Positivo	Negativo	9,13 x101	Negativo
PEP 32B	PEP	M	Adulto	Negativo	Negativo	Negativo	Positivo	Negativo	Negativo	1,04x103	Negativo
PEP 49	PEP	M	Filhote	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo
PEP 01D	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,18x103	Negativo
PEP 43B	PEP	M	Adulto	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	1,58x103	Negativo
PEP 50	PEP	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	5,49x103	Negativo
PEP 51	PEP	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	5,22x103	Negativo
VBA 07D	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	2,96x102	Negativo
VBA 09D	VBA	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo

VBA 11C	VBA	M	Adulto	Negativo	Positivo	Negativo	Positivo	Negativo	Negativo	7,11x103	Negativo
VBA 03D	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	3,49x102	Negativo
VBA 21C	VBA	M	Subadulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	3,00x102	Negativo
VBA 39B	VBA	F	Adulta	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	5,14 x101	Negativo
VBA 23B	VBA	F	Adulta	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	4,74x100	Negativo
VBA 29B	VBA	M	Filhote	Negativo	Negativo	Negativo	Positivo	Negativo	Negativo	2,18x102	Negativo
VBA 41	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	2,05x103	Negativo
VBA 42	VBA	M	Filhote	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	8,13 x101	Negativo
VBA 25C	VBA	M	Filhote	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,70 x101	Negativo
VBA 43	VBA	M	Adulto	Negativo	Positivo	Negativo	Positivo	Negativo	Negativo	2,56x100	Negativo
VBA 06B	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	5,74x102	Negativo
VBA 44	VBA	M	Adulto	Negativo	Positivo	Negativo	Positivo	Negativo	Negativo	2,09x102	Negativo
VBA 16C	VBA	F	Adulto	Negativo	Positivo	Positivo	Negativo	Positivo	Negativo	2,62 x101	Negativo
VBA 39C	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 41B	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	2,33x102	Negativo
VBA 45	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 29C	VBA	M	Subadulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	4,65x102	Negativo
VBA 46	VBA	F	Filhote	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	5,33x102	Negativo
VBA 47	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	2,96x102	Negativo
VBA 48	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,88 x101	Negativo
VBA 03E	VBA	F	Adulto	Negativo	Negativo	Negativo	Positivo	Negativo	Negativo	1,55x102	Negativo
VBA 49	VBA	F	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,02x102	Negativo
VBA 05D	VBA	M	Adulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	1,70x102	Negativo
VBA 17B	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	9,79 x101	Negativo
VBA 25D	VBA	M	subadulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	3,33x100	Negativo
VBA 21D	VBA	M	subadulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	4,94x100	Negativo
VBA 50	VBA	M	Filhote	Negativo	Negativo	Negativo	Negativo	Positivo	Negativo	6,72x100	Negativo
VBA 52	VBA	F	subadulto	Positivo	Negativo	Negativo	Negativo	Positivo	Negativo	1,41x103	Negativo
VBA 03F	VBA	F	Adulto	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	4,27 x101	Negativo
VBA 21E	VBA	M	Subadulto	Negativo	Positivo	Negativo	Positivo	Negativo	Negativo	5,39 x101	Negativo

VBA 53	VBA	F	Adulto	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo	2,85x100	Negativo
VBA 44B	VBA	M	Adulto	Positivo	Positivo	Negativo	Positivo	Negativo	Negativo	2,38 x101	Negativo
VBA 54	VBA	M	Subadulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	2,39x102	Negativo
VBA 55	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	Negativo
VBA 38B	VBA	F	Subadulto	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	2,03x102	Negativo
VBA 56	VBA	F	Subadulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	3,18x100	Negativo
VBA 57	VBA	F	Adulto	Positivo	Positivo	Negativo	Positivo	Negativo	Negativo	2,90x100	Negativo
VBA 16D	VBA	F	Adulto	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	1,31 x101	Negativo
VBA 25E	VBA	M	Subadulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,10x102	Negativo
VBA 08C	VBA	F	Adulto	Negativo	Positivo	Negativo	Positivo	Negativo	Negativo	9,03x103	Negativo
VBA 58	VBA	M	Filhote	Positivo	Negativo	Negativo	Negativo	Negativo	Negativo	8,98x102	Negativo
VBA 21F	VBA	M	Subadulto	Positivo	Negativo	Negativo	Positivo	Negativo	Negativo	5,98x104	Negativo
VBA 59	VBA	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	3,08x102	Negativo
VBA 19B	VBA	F	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	5,48 x101	Negativo
VBA 60	VBA	M	Subadulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,58x102	Negativo
VBA 01B	VBA	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	1,36 x101	Negativo
VBA 44C	VBA	M	Adulto	Positivo	Positivo	Negativo	Negativo	Negativo	Negativo	1,88 x102	Negativo
VBA 55B	VBA	M	Adulto	Negativo	Positivo	Negativo	Negativo	Negativo	Negativo	2,09 x102	Negativo