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Original Article

Is there only one species of *Hepatozoon* infecting Brazilian caimans? Integrative taxonomy unveiling the parasite's diversity

Há somente uma espécie de *Hepatozoon* infectando caimans brasileiros? Taxonomia integrativa desvendando essa diversidade parasitária

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Abstract

Hepatozoon spp. are the most common haemoparasites reported from reptiles around the world, however, only six species have been described infecting crocodilians. In Brazil, *Hepatozoon caimani* Carini, 1909 is currently the only recognized species from the caiman hosts. This study provides new data on the diversity of species of *Hepatozoon* infecting *Caiman crocodilus* (Linnaeus) using molecular data and phylogenetic analysis, with additional support of morphological data of developmental stages from host blood and tissue. Forty-four individuals were collected and screened for haemogregarines, and blood and tissue samples were analysed by light microscopy with 31 (70.45%) infected. *Hepatozoon* spp. blood developmental stages included immature and mature gamonts with or without cytoplasmic vacuoles and free gamonts. Additionally, merogonic developmental stages were found in the liver and spleen of infected hosts. Based on the morphological and molecular data, this study identified two possible different species of *Hepatozoon*, being one of them the *H. caimani* with intragenotypic divergence.

Keywords: Hemogregarine, crocodilian, Brazil, diversity, PCR.

Resumo

Hepatozoon spp. são os hemoparasitas mais comuns relatados em répteis em todo o mundo, no entanto, apenas seis espécies foram descritas infectando crocodilianos. No Brasil, *Hepatozoon caimani* Carini, 1909 é atualmente a única espécie reconhecida dos hospedeiros jacarés. Este estudo fornece novos dados sobre a diversidade de espécies de *Hepatozoon* que infectam *Caiman crocodilus* (Linnaeus) utilizando dados moleculares e análise filogenética, com suporte adicional fornecido através de dados morfológicos de estágios de desenvolvimento do sangue e tecido do hospedeiro. Quarenta e quatro indivíduos foram coletados e triados para hemogregarinas, e amostras de sangue e tecidos foram analisadas por microscopia óptica com 31 (70,45%) infectados. Os estágios de desenvolvimento do sangue incluíram gamontes imaturos e maduros com ou sem vacúolos citoplasmáticos e gamontes livres. Além disso, foram encontrados estágios de desenvolvimento merogônico no fígado e baço de hospedeiros infectados. Com base nos dados morfológicos e moleculares, este estudo identificou duas possíveis espécies diferentes de *Hepatozoon*, sendo uma delas, o *H. caimani* com divergência intragenotípica.

Palavras-chave: Hemogregarina, crocodilianos, Brasil, diversidade, PCR.

1. Introduction

Haemogregarines (Apicomplexa: Adeleiorina) are the most common and widely distributed reptilian haemoparasites (Telford Junior, 2009). These heteroxenous apicomplexan parasites are found infecting the blood cells of a wide range of vertebrate and invertebrate hosts from all orders and are known to parasitize living reptiles (Telford Junior, 1984; Smith, 1996; Telford Junior et al., 2004). Haemogregarines are currently divided into the families Dactylosomatidae Jakowska and Nigrelli, 1955, Haemogregarinidae Léger, 1911, Hepatozoidae Miller, 1908, and Karyolysidae Labbé, 1894 (Netherlands et al., 2018). Concerning crocodilian hosts, for many years all haemogregarines recorded in Brazil

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were assigned to the genus *Haemogregarina* Danilewsky, 1885 (Apicomplexa: Adeleoria). However, Smith (1996) reassigned these species to the genus *Hepatozoon* (Miller, 1908), except for *Haemogregarina brasiliensis* Di Primo, 1925 from *Caiman yacare* found in Mato Grosso State, Brazil (Smith, 1996; Duszynski et al., 2020).

Currently, the genus *Hepatozoon* has the highest number of species (Smith, 1996). However, according to a recent revision on coccidian parasites infecting crocodilians of the world (Duszynski et al., 2020), there are currently only six species described in crocodilian hosts, as follows: *Hepatozoon hankini* Simond, 1901 described from *Gavialis gangeticus* (Gmelin) in Asia (India); *Hepatozoon serrei* Phisalix, 1914 reported from *Paleosuchus trigonatus* (Schneider) in South America; *Hepatozoon petiti* (Thiroux, 1910) Hoare 1932 infecting *Crocodylus niloticus* Laurent in Africa; *Hepatozoon sheppardi* Santos Dias, 1952 found parasitizing *C. niloticus* in Africa; *Hepatozoon crocodilinorum* (Borner, 1901) Smith, 1996 infecting *Alligator mississippiensis* (Daudin) and *Osteolaemus tetraspis* Cope from Europe and North America; and *Hepatozoon caimani* Carini, 1909, from Brazilian caimans, *Caiman latirostris* (Daudin), *Caiman yacare* (Daudin), and *C. crocodilus*.

In Brazil, *H. caimani* is the only valid species of *Hepatozoon* in Brazilian caimans, with studies mainly focused on morphological descriptions, parasite life history, and occurrence. High prevalence of *H. caimani* has been reported, for example, 76.7% in *C. crocodilus* from the Amazon region and 71.4% of *C. yacare* in from the western were infected, and 76% – 79.5% of caimans from southeastern Pantanal (Viana and Marques, 2005; Viana et al., 2010; Soares et al., 2017). This high prevalence could be related to the number of transmission routes available for *H. caimani* to its caiman hosts, via ingestions of infected mosquitoes (vector), infected frog, fish, snakes and small alligators (intermediate hosts (Lainson et al., 2003; Pereira et al., 2014).

Telford Junior et al. (2004) proposed the use of molecular tools as the best way to detect species of *Hepatozoon* and infer taxonomic relationships among the species that infect a wide range of caiman species. To date, only three studies (Clemente et al. 2023; Bouer et al. 2017; Soares et al., 2017) targeting the 18S rRNA gene, have molecularly identified *Hepatozoon* from caimans in Brazil. Moreover, the recently published article by Clemente et al. (2023) analyzed 111 reptile samples from the state of Mato Grosso, Brazil, with molecular diagnosis targeting the 18S SSU gene. The authors reported isolates of *Hepatozoon caimani* in *Caiman yacare* and *Paleosuchus palpebrosus*.

Therefore, this study aimed to compare literature available data with the new data obtained in present study, to highlight the diversity of species of *Hepatozoon* in Brazilian caimans, using molecular, phylogenetic, morphological, and morphometric analyses.

2. Material and Methods

2.1. Host collection

During fieldworks conducted from 2017 to 2020, Brazilian caimans were collected from Mato Grosso State, Brazil, in two localities: Boa Esperança Farm (14°46'44.82" S, 51°32'50.86" W) and Sol Vermelho Farm (14°28'39.27" S, 51°36'31.95" W), both in the municipality of Cocalinho, Mato Grosso state, Brazil.

The animals were submitted to physical restraint with the appropriate equipment, following standard procedure for this group of animals. The crocodilians were captured by active sampling using the hands or fishing rods with steel guy wire. Restraint was performed by sealing the eyes and mouth with tape and tying the paws with rope (Santos et al., 2011; Viana et al., 2010). Before the blood collection, the animals were submitted to sexing, measured, and weighed for classification of the crocodilian age group, according to Velasco et al. (2009): hatchlings (length ≤ 25cm), juveniles (25-50cm), young adults (51-80cm), and adults (≥80cm).

Blood samples were collected by puncture of the cervical paravertebral sinus using sterile and disposable syringes and needles (Zippel et al., 2001) Three thin blood smears were made and the remaining blood sample was stored in EDTA tubes and frozen at -10ºC for further molecular analysis. The blood smears were fixed with absolute methanol and stained with 10% Giemsa Methylene Blue Eosin Merck® diluted in distilled water (pH 7.0) for 50 min, according to Eisen and Schall (2000). For histological slides, six caimans were euthanized using 50 mg/kg sodium thiopental (Tiopentax®) administered intracerebrally, following the guidelines of Sebben (2007) and the Animal Ethics Committee of Veterinary Medicine. The liver, spleen, heart, and kidney were fixed in 4% buffered neutral formalin and stained with hematoxylin-eosin (Eisen and Schall, 2000).

All applicable international, national, and institutional guidelines for the ethical handling of animals were followed (SISBIO license 60640-1; CEUA-UNESP 1061).

2.2. Morphological study of the parasites

For morphological analysis of the blood and tissue parasite stages, digital images were captured and measured using a compound microscope at 100x magnification with the Leica software application suite LAS V3.8 (Leica Microsystems). Measurements are in micrometres (µm) comprising the parasite's length and width, with mean and standard deviation (means ± standard deviation) given. Parasitaemia was calculated with ~10^4 erythrocytes examined per blood smear following Cook et al. (2009).

2.3. Molecular analyses

DNA was extracted from blood samples following the DNeasy Blood & Tissue Kit standard protocol (Qiagen, Valencia, CA, USA). Two PCR assays were performed targeting two different regions of the parasites 18S rDNA using the HepF300 and Hep900 pair of primers, which amplifies 600 bp (Ujvari and Marques, 2005) and Hemo1 and Hemo2 pair of primers, which amplifies 900 bp (Perkins and Keller, 2001) PCR amplification reactions were carried out in a final volume of 25 µL, containing 1 µL each of 10 pmol primers, 12.5 µL of Master Mix MyFiTM Mix Bioline®, and 5 µL of extracted DNA, with nuclease-free water accounting for the remaining volume. PCR amplification was performed on a Peltier 200 Thermocycler (MJ Research, Watertown, MA) (O'Dwyer et al., 2013).

PCR products were subjected to gel electrophoresis at 80 V in a 1.5% agarose gel, stained with Gel Red, and observed using an ultraviolet transilluminator. The products of interest were purified by adding 2 µL of ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) to 5 µL of PCR product according to the manufacturer's recommendations. Amplicons were then sequenced, using PCR primers on a 3,500 Genetic Analyzer capillary sequencer (Applied Biosystems) and after BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3.1 (Applied Biosystems) according to the manufacturer's recommendations. A consensus sequence was created from the forward and reverse-assembled electropherograms using Geneious version 7.1.3 (Kearse et al., 2012). For each positive animal, the newly generated sequences from HepF300/Hep900 and Hemo1/Hemo2 contigs were concatenated using Geneious version 7.1.3, forming a longer sequence of ~1,200 bp.

The sequences from this study were compared with other isolates from haemogregarine parasites available at GenBank. The newly generated sequences of partial 18S rDNA were aligned using Geneious version 7.1.3 with the Muscle algorithm implemented from within Geneious version 7.1.3 (Bomatters, www.geneious.com) and default settings with related sequences that appeared on Blastn search.

Phylogenetic reconstructions were performed using the Bayesian inference (BI) and Maximum Likelihood (ML) methods. The BI analysis was carried out using MrBayes implemented from the computational resource CIPRES (Miller et al., 2010), and the analysis was run with the nucleotide substitution model GTR+I+G. The Markov chain Monte Carlo (MCMC) algorithm was run with 10,000,000 generations, saving one tree every 1000 generations. The first 25% of the trees were discarded as burn-in, and the consensus trees were estimated using the remaining trees. Bayesian posterior probabilities (BPP) cut-off was considered > 50%. The trees were visualized and edited using the FigTree v1.4 software program (Rambaut, 2012). For the ML method, based on the Akaike information criterion (AIC) the Transitional model (Posada, 2003), a discrete Gamma distribution (TVM+G) was selected. Phylogenetic analysis was inferred using PhyML (Guindon and Gascuel, 2003) with 1,000 bootstrap replicates (>50%).

Isolates from other adeleorinid parasites (Haemogregarinidae, Hepatozoidae, Karyolysidae, and Dactylosomatidae) available from GenBank were used to construct both phylogenetic trees. The obtained phylogenetic trees for the BI and ML analyses were edited in FigTree v1.4 (Rambaut, 2012). *Lankesterella minima* (Chaussat 1850) (GenBank: AF080611) and *Isospora wiegmanniana* Megía-Palma et al., 2015 (GenBank: KU180242) from the Order Eucoccidiorida were selected as out-group. A pair-wise distance (p-distance) matrix was used to compare the interspecific divergence between species of *Hepatozoon* sequences isolated from caimans hosts.

3. Results

A total of 44 *Caiman crocodilus* was screened and 31 (70.45%) were infected with species of *Hepatozoon*, based on morphological screening of the blood smears. The parasitaemia ranged from 0.01% - 6% in the infected caimans. No ectoparasites, such as leeches, were observed infesting the caimans.

With regards to the prevalence and gender of the crocodilians, 75.86% (22/29) of the males and 63.63% (7/11) of the females were positive. The gender of four caimans was undetermined; of these, two were positive.

Regarding age, 70.31% (23/29) of adults, 55.50% (5/9) of young adults, and 50% (3/6) of juvenile crocodilians were found positive.

3.1. Molecular data

All 31 specimens found positive through morphological screening had their *Hepatozoon* spp. successfully amplified using PCR and sequencing targeting the 18S rRNA gene. The newly generated and concatenated sequences were compared to each other and with other *Hepatozoon* isolates from crocodilians available on GenBank. The genetic divergence of 98.35% – 100% was obtained for species of *Hepatozoon* isolated from caiman hosts in the present study and compared with sequences from GenBank (Table 1).

In the phylogenetic tree, two main clades were observed (Figure 1). The first comprised species of *Dactylosoma* Labbé, 1894 and *Haemogregarina*, and the second comprised *Hemolivia* Petit, Landau, Beccam and Lainson, 1990, *Karyolysus* Labbé, 1894 *Hepatozoon* (mammals), and *Hepatozoon* (reptiles and amphibians) clades. Regarding the latter clade, two subclades were recovered, one with species of *Hepatozoon* isolated from reptiles and anurans hosts formed a sister group to the second larger subclade comprising isolates from crocodilian hosts.

Table 1. The shaded matrix (lower) shows the percentage of similarity of the 18S rDNA nucleotide sequences and the non-shaded matrix (upper) shows the p-distance (pair-wise distance) between the *Hepatozoon* sequences isolated from caiman hosts and compared to sequences available on GenBank.

	1.	2.	3.	4.	5.	6.
1. Hepatozoon caimani (C1)		0.010	0.006	0.014	0.013	0.010
2. Hepatozoon caimani (C2)	98.98%		0.009	0.012	0.011	0.011
3. Hepatozoon caimani (C3)	99.39%	99.39%		0.016	0.018	0.014
4. Hepatozoon sp. (B)	98.78%	98.45%	98.54%		0.014	0.010
5. Hepatozoon sp. (A1)	98.78%	98.78%	98.35%	98.61%		0.006
6. Hepatozoon sp. (A2)	99.13%	99.13%	98.70%	99.13%	99.30%	

Phylogenetic position of the clades and subclades of *Hepatozoon* spp. from caimans hosts (C1: subclade C1. C2: subclade C2. C3: subclade C3. B: subclade B. A1: subclade A1. A2: subclade A2).

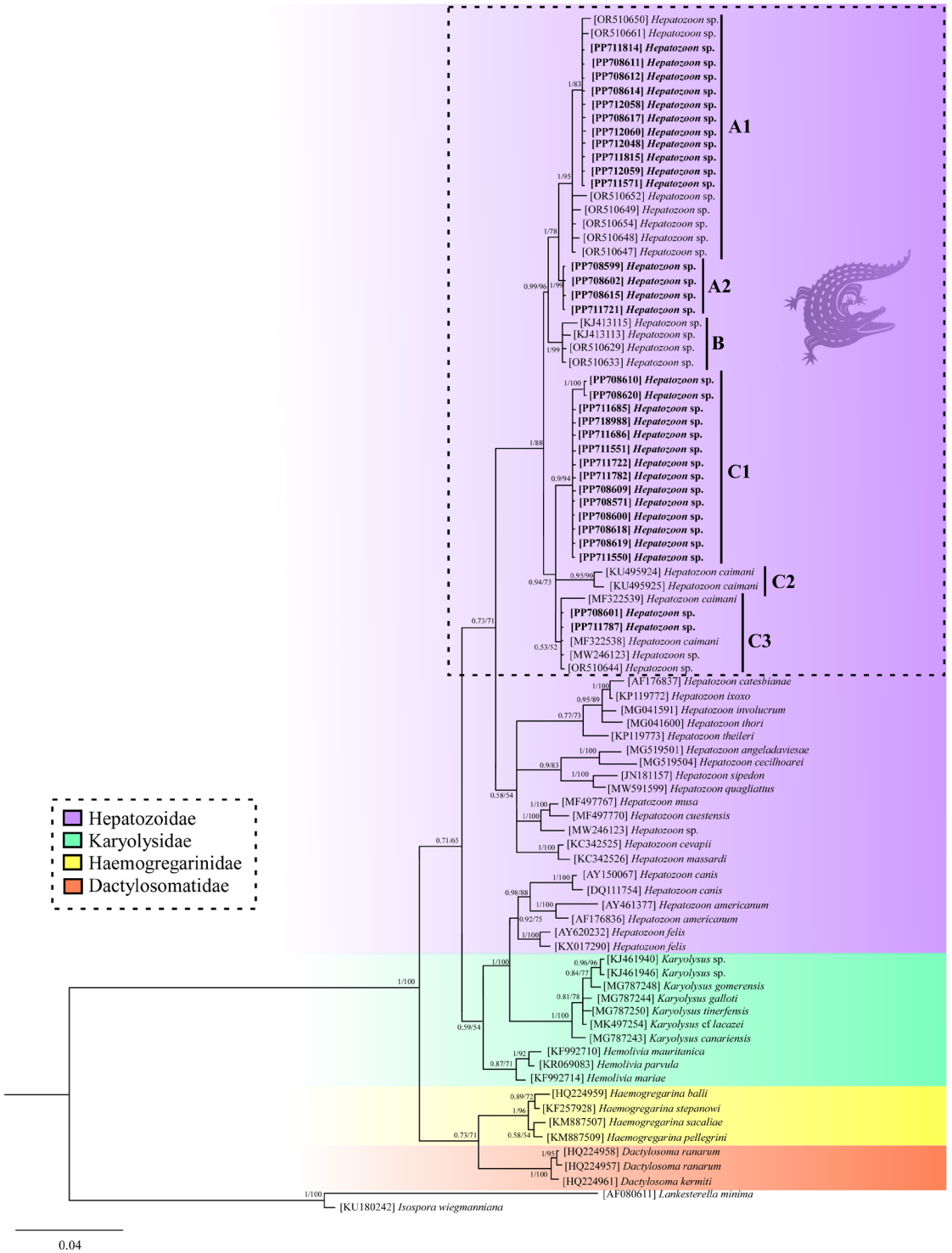

Figure 1. Consensus phylogram of hemogregarines based on 18S rDNA sequences. The topology trees were inferred by Bayesian (BI) and Maximum Likelihood (ML) methods (represented by ML tree). The isolates *Isospora wiegmanniana* (KU180242) and *Lankesterella minima* (AF080611) were used as an out-group.

The crocodilian clade forms a monophyly that represents a polytomy with two well-supported branches. In the first branch, two subclades were observed. The subclade A (A1 and A2), the A1 were observed comprising 11 isolates from the present study with 100% gene identity, with a polytomy with seven isolates of *Hepatozoon* sp. from *C. yacare* (Clemente et al., 2023); and A2 consisting of four isolates from the present study with similarities ranging from 99.8% to 100%.

The branch B comprises sequences of two isolates of *Hepatozoon* sp. (KJ413115 and KJ413113) from *C. yacare,* and two sequences of *Hepatozoon caimani* from *C. yacare* (OR510629 and OR510633) (Clemente et al., 2023) with 99.6% similarity (18S rDNA) among them.

In regards to branch C (C1, C2 and C3), the C1 has shown 99.74% similarity among the sequences, being two identical isolates (R66, R117) with another 12 identical isolates, all from the present study. The C2 is composed of two *H. caimani* isolates (KU495924, KU495925) from *C. yacare*, with 99% similarity between these isolates. The branch C3 comprises *H. caimani* (MF322539, MF322538) from *Caiman crocodilus yacare* Wermuth & Mertens, 1977 obtained from GenBank, one isolate (MW246123) of *C. crocodilus* from Colombia, one isolate from *C. yacare* (OR510644) and two isolates from the present study (R46, R71) with 99.8% - 100% of gene similarity.

Thus, the molecular data analysis revealed the presence of *H. caimani* (Branch C) and one unidentified species of *Hepatozoon* (Branch A) in Brazilian caimans from the present study, all with intragenotypic divergences. In regards to *H. caimani* genotypes, the branches C contained intraspecific gene similarity of 99.74% (pair-wise distance of 0.006) and an interspecific gene similarity ranging between 98.35% – 98.96% as compared to the other genotypes from Brazilian crocodilians. In regards to the undescribed species, branch A1 have shown an interspecific gene similarity ranging from 98.45% to 99.30% (pair-wise distance of 0.018 – 0.006) compared to the other isolates from crocodilians. The second genotype from the undescribed species (branch A2) has shown an interspecific gene similarity ranging between 98.70% – 99.30% (pair-wise distance of 0.006 – 0.0014) as compared to the other isolates of Brazilian crocodilians.

3.2. Morphological data

The morphological data obtained from the blood smears of the different collected specimens revealed diverse morphological characteristics (Figures 2-5). Moreover, initial merogonic developmental stages were detected in the histological slides of the spleen and liver, from the five-euthanized caimans (Figures 3 and 4). The morphometric data are reported in Table 2.

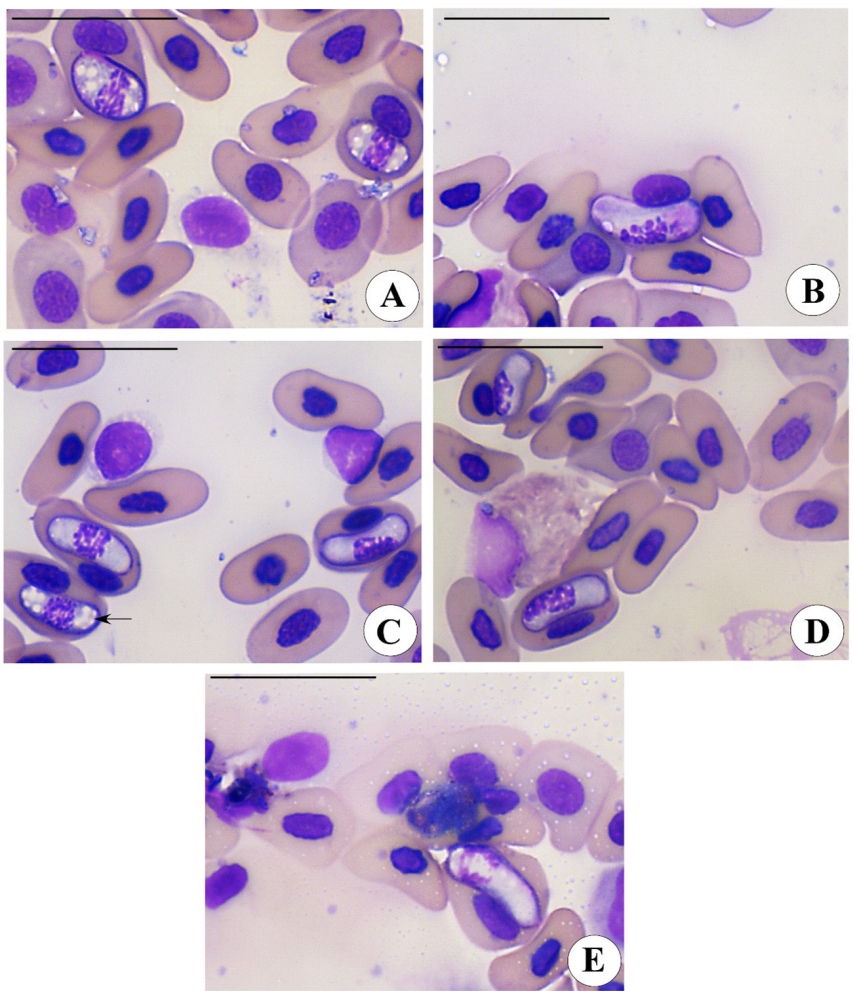

Figure 2. A-E. Morphological data on *Hepatozoon caimani* (genotype C3) in two caimans blood smears from Mato Grosso State, Brazil. **A-B)** Gamonts with cytoplasmic vacuoles; **C-D)** Mature gamonts and gamonts with cytoplasmic vacuoles (arrow); **D)** Gamonts; **E)** Gamont with whitish cytoplasm and capsule stained in dark-purple. Scale bar: 20µm

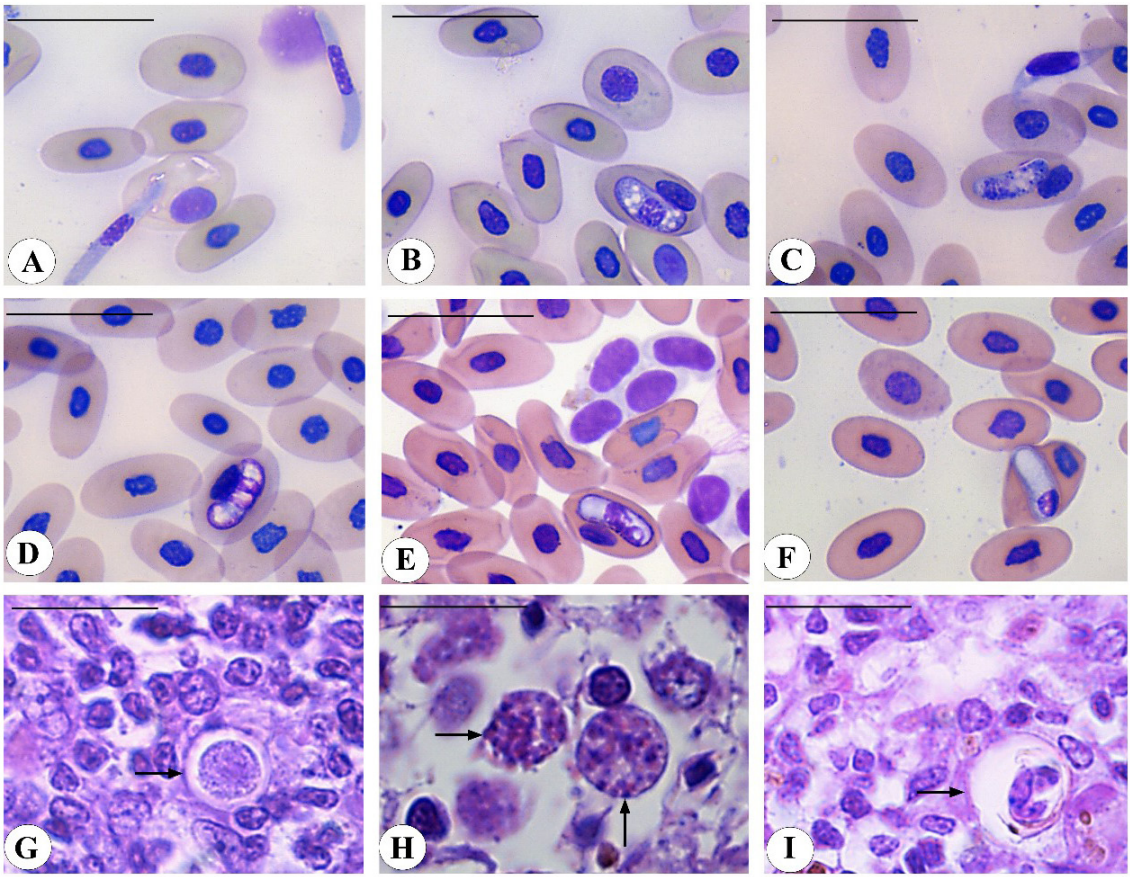

Figure 3. A-I. Morphological data on *Hepatozoon caimani* (genotype C1) in 14 caimans blood smears from Mato Grosso State, Brazil. **A)** Free-gamonts; **B)** Stout immature gamonts; **C)** Immature gamonts with cytoplasmic and nuclei chromatin granules loosely arranged across thee parasites cytoplasm; **D)** Late immature gamonts with slender and spread chromatin nuclear through the cytoplasm; **E-F)** Mature gamonts; **G)** Early-stage meront in the caiman´s spleen; **H-I)** Meronts with merozoites in the spleen. Scale bar 20µm.

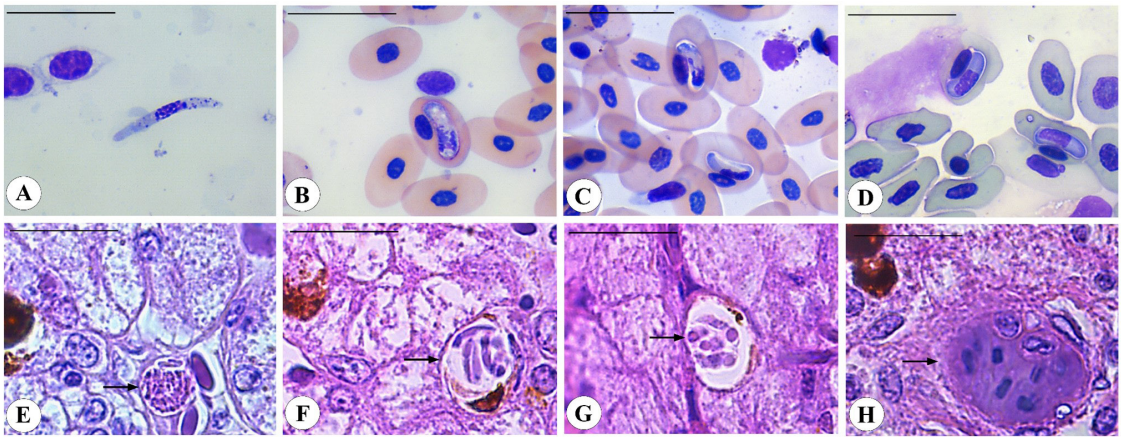

Figure 4. A-H. Morphological data on *Hepatozoon* sp. (genotype A1) in 11 caimans blood smears from Mato Grosso State, Brazil. **A)** Free-gamonts; **B)** Stout immature gamonts with cytoplasmic vacuoles; **C)** Immature gamont with dispersed nuclear chromatin; **D)** Mature gamonts; **E)** Micromeronts in the liver; **F)** meronts with merozoites in the liver; **G)** granule-mass structure meront, considered as an early stage of development; **H)** macromeronts with macromerozoites in the spleen. Scale bar 20µm

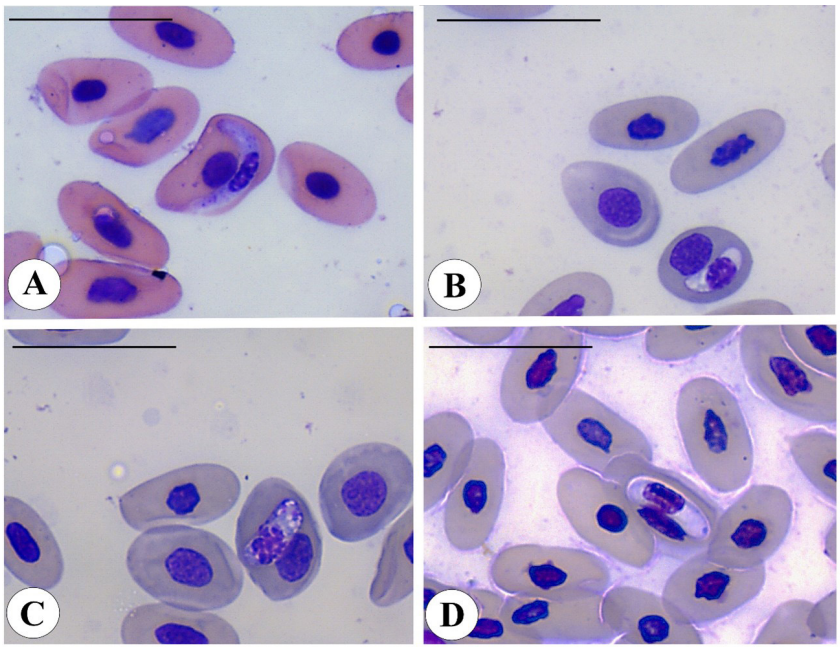

Figure 5. A-D. Morphological data on *Hepatozoon* sp. (genotype A2) in four caimans blood smears from Mato Grosso State, Brazil. **A)** Free-gamont infecting the erythrocyte; **B)** Trophozoite; **C)** Immature gamonts with cytoplasmic granules; **D)** Mature gamonts. Scale bar 20µm.

The morphological characteristics of *H. caimani* (Branch C3) observed in the present study were immature (small) gamonts with vacuoles, mature (larger) gamonts with vacuoles, mature gamonts, and gamonts with slender nuclear chromatin with a whitish cytoplasm (Figure 2). Parasitaemias were 0.5% and 6% in the two infected individuals.

The morphological characteristics of *H. caimani* (branch C1) observed in the present study were the presence of free gamonts, immature gamonts, mature gamonts and meronts in the spleen and liver (Figure 3). Free gamonts (Figure 3A) were long and thin parasites with the nuclei with dense purplish chromatin located centrally. In relation to immature gamonts (Figure 3B-E), three different morphological stages were observed.The first one, a stout immature gamont (Figure 3B) presented cytoplasmic vacuoles, always displacing cell nuclei to one side, with rounded and dense nuclei located centrally of the parasite's body. In the other morphology, the gamonts have cytoplasmic and nuclei chromatin granules loosely arranged across the parasite cytoplasm (Fig, 3C), with the presence or not of vacuoles. The nuclear chromatin is stained in purple, while the granules are dark blue. It is possible to observe that the cell nuclei are always dislocated to one extremity. The third morphology, considered as late immature gamonts (Figure 3D), is characterised by gamonts with slender and spread chromatin nuclear through the cytoplasm, with vacuoles and whitish cytoplasm. A dark purple stain can be observed through the parasite's membrane and nuclei. Two morphotypes of mature gamonts (Figure 3E-F) were evidenced; one with widespread nuclei (Figure 3E), occupying 2/3 of the parasite's body, with slender nuclear chromatin stained in purple. The parasite's membrane is stained in dark purple and vacuoles can or cannot be observed. The parasite's body ends are rounded and always displacing the cell nuclei to one side. A slightly bigger parasite's body characterizes the second morphotype (Figure 3F), with smaller, rounded, and dense nuclei always dislocated to the curved extremity of the parasite's body. It is possible to observe a significant displacement of the nuclei cell and damaged structures of the erythrocyte in this case. The parasite's membrane is stained in the same colour as the cytoplasm. Meronts in different stages of development were detected in the spleen and liver (Figure 3G-I). The earlier stage had only an internal mass (Figure 3G) whereas the mature stage presented formed merozoites (Figure 3H, I). All the meronts had an oval and rounded body, with the capsule stained in dark purple (Figure 3H) or whitish (Figure 3G, I). The total prevalence found was 31.81% (14/44), and parasitaemia ranged from 0.1% to 1%.

The undescribed species of *Hepatozoon* (Subclade A), through morphological analysis, revealed developmental stages in the peripheral blood and tissue fragment of the liver. The total prevalence found was 36,36% (16/44), and the parasitaemia ranged from 0.01% to 6%. All measurements of the different parasite stages are available in Table 2.

In regards to the morphological data, the trophozoite (Figure 5B) presented a small shape with one side more tapered than the other. The rounded purple nuclei are located at the centre of the parasite. A slight displacement of the nuclei cell could be observed in some cases. The immature gamonts (Figure 4C) were encased in a visible parasitophorous vacuole, with nuclear chromatin loosely spread across the parasite's cytoplasm, or located to one side of the parasite (probably dependent on the timing of development). Nuclear chromatin staining dark purple. Sometimes, cytoplasmic vacuoles could be observed. The gamonts have both ends rounded with one slightly more

Table 2. *Hepatozoon* spp. measurements (mean ± standard deviation) of blood and tissue stages in crocodilians, *Caiman crocodilus*, from Brazil.

Hepatozoon	Developmental stages	N	C (µm)	PL (µm)	PW (µm)	PA (µm²)	NL (µm)	NW (µm)	NA (µm²)
H. caimani Genotype C3 (Figure 2)	IGV	15		9.82 ± 2.01	5.45 ± 0.56	47.08 ± 2.53	3.74 ± 0.55	5.41± 0.38	11.02 ± 0.85
	GV	15		13.85 ± 0.99	5.10 ± 0.79	61.95 ± 3.99	5.35 ± 0.21	4.14 ± 0.61	12.67 ± 0.97
	MG	25		14.75 ± 1.85	5.71 ± 1.33	59.64 ± 2.14	4.18 ± 0.74	3.79 ± 1.00	16.01 ± 1.00
	GS	2		17.51± 0.58	6.43 ± 0.44	94.11 ± 1.80	5.85 ± 1.01	5.04 ± 0.92	18.07 ± 1.32
H. caimani	FG	25		24.71 ± 1.06	3.42 ± 0.96	59.40 ± 6.01	6.41 ± 0.42	3.09 ± 0.61	12.07 ± 1.33
Genotype C1	IGV	30		13.95 ± 1.21	4.77 ± 1.00	62.53 ± 8.05	5.45 ± 0.98	3.93 ± 0.76	12.75 ± 2.03
(Figure 3)	IGG	15		13.87 ± 1.21	5.02 ± 0.99	64.78 ± 0.86	6.78 ± 1.72	4.87 ± 1.01	16.78 ± 3.05
	LIG	50		13.59 ± 1.43	3.54 ± 0.86	55.64 ± 13.36	5.98 ± 1.26	3.20 ± 0.48	13.30 ± 2.99
	MG1			15.57 ± 1.72	5.84 ± 0.98	77.93 ± 5.01	4.67 ± 0.87	4.05 ± 1.23	14.54 ± 2.00
	Mi	15	3.82 ± 0.41	9.26 ± 0.85	9.90 ± 1.30	73.58 ± 19.55			
	Miz	10		4.80 ± 0.94	1.20 ± 0.22	5.36 ± 1.02	0.82 ± 0.68	1.06 ± 0.41	1.67 ± 0.55
Hepatozoon sp.	FG	15		25.28 ± 0.70	2.73 ± 0.56	61.38 ± 5.10	8.77 ± 0.65	2.73 ± 0.56	14.60 ± 1.75
Genotype A1	IG	20		14.70 ± 2.01	5.12 ± 3.0	70.88 ± 2.74	4.44 ± 0.71	4.77 ± 0.97	16.08 ± 2.54
(Figure 4)	MG	40		13.47 ± 0.46	4.55 ± 0.40	55.75 ± 3.91	6.75 ± 0.62	2.79 ± 0.36	15.43 ± 1.78
	Mi	16	1.75 ± 0.31	10.81 ± 2.00	9.69 ± 1.68	76.93 ± 9.41			
	Ma (R109)	2	N - 4.45	14.05 - 13.32	16.20 - 19.16	142.60 – 169.12			
	Maz	5		7.56 ± 0.40	1.82 ± 0.25	11.03 ± 1.55	2.60 ± 0.38	1.68 ± 0.25	3.67 ± 0.59
	Ma (R115)	1		29.39	22.35	366.06			
	Maz	7		9.27 ± 0.65	3.39 ± 0.20	18.90 ± 3.20	3.80 ± 0.63	2.15 ± 0.19	9.22 ± 1.31
Hepatozoon sp.	FG	25		12.83 ± 1.51	4.35 ± 0.33	51.66 ± 0.62	6.37 ± 0.92	3.33 ± 0.54	12.01 ± 1.85
Genotype A2	T	25		10.19 ± 0.83	3.88 ± 1.02	42.81 ± 3.78	5.93 ± 1.24	2.86 ± 0.91	12.04 ± 0.79
(Figure 5)	IG	50		12.83 ± 1.51	4.35 ± 0.33	51.66 ± 4.02	6.37 ± 0.92	3.35 ± 0.54	14.97 ± 2.00
	MG	50		12.84 ± 0.34	4.88 ± 0.17	61.12 ± 2.09	6.46 ± 0.62	4.24 ± 0.43	15.04 ± 1.22

C: meront´s capsule; N: number of parasites PL: parasite length; PW: Parasite width; PA: Parasite area; NL: Parasite nuclei length; NW: Parasite nuclei width; NA: Parasite nuclei area. FG: free-gamonts. IGV: immature gamonts with cytoplasmic vacuoles. GS: gamonts with slender nuclear chromatin and whitish cytoplasm. GV: gamonts with cytoplasmic vacuoles. MG: mature gamonts. LIG: Mature gamonts with bigger nuclei and parasite´s membrane stained in dark purple. MG1: slightly bigger gamont with smaller nuclei. IGG: immature gamonts with cytoplasmic granules and nuclear chromatin sparse through the parasite´s body. IG: immature gamont. T: trophozoites. Mi: micromeront. Miz: micromerozoite. Ma: macromeront. Maz: macromerozoite. N: meronts capsule non-evidenced.

curved than the other. Displacement of the cell nuclei is always present. The stout immature gamont (Figure 4B; 5C) were found in two morphologies. The first (Figure 4B) is characterised by lighter-purple nuclear chromatin loosely arranged and located towards one side of the parasite with the presence of cytoplasmic vacuoles. Both ends of the parasite were rounded. The host cell nucleus is always displaced to one side of the erythrocyte. In some cases, it is possible to observe cytoplasmic granules. The second morphology (Figure 5C) is characterised by the presence of cytoplasmic vacuoles, and in some cases, it is possible to observe some granules. The purple and round nuclei are dislocated to one side of the parasite, which has both extremities rounded. The displacement of the nuclei cell to one side of the erythrocyte is also evidenced. The Mature gamonts (Figure 4D; 5D) were found in two morphologies. The first one (Figure 4D) is characterised by shape and size consistent with an evident parasitophorous vacuole. Both sides of the parasite are rounded, with one more arched than the other. The nuclei are always situated on the arched side of the parasite, staining purple with an oblong shape, occupying half of the parasite's body. The parasite-curved side is always located close to the displaced host cell nucleus. The parasite's cytoplasm is stained light purple. The second morphology found (Figure 5D) has dense and rectangular nuclei, slightly displaced to one side of the parasite body, stained in dark purple. Sometimes, a small, rounded, stained-in-purple structure located at the opposing side of the parasite's nuclei was observed. The parasite has both ends rounded with a thin capsule evidenced, and always located close to the cell nuclei. The free-gamonts (Figure 4A; 5A) were characterised by long and thin body with cytoplasmic vacuoles and granules, nuclei staining purple and located centrally to the parasite's body. The nuclei are oval, centrally positioned,with dark-purple chromatin stained. Sometimes, it was possible to observe this type of gamont inside the erythrocyte. Meronts in different merogonic stages were observed in the liver and spleen tissues (Figure 4E-H) Micromeronts (Figure 4E, F), meronts with granule-mass structure, considered as an early-stage development (Figure 4G), and macromeronts (Figure 4H) were detected. In the micromeronts and macromeronts, merozoites could be observed. Oval to rounded shapes were presented with a capsule not always evidenced.

4. Discussion

Crocodilians are large, long-lived predators, with a generalist predatory behaviour, being able to accumulate various trophically transmitted parasites through the consumption of a variety of prey over an extended period (Tellez, 2013; Duszynski et al., 2020). This ability can be related to the easy habitat transition to aquatic and terrestrial during their lifetime, increasing the variety of pathogens and their vectors' contact (Tellez, 2013). Therefore, the understanding of the ecological and host-prey relationship among caimans during their life timing is essential for the parasite's prevalence and parasitaemia correlation. It is known that young animals, in their first year of life, feed essentially on invertebrates, particularly arthropods (Uetanabaro, 1989; Viana et al., 2010). From the second year on, juvenile and adult caimans start to feed on anurans and fishes, which form an important transmission phase through predation of intermediate hosts (Uetanabaro, 1989; Viana et al., 2010). In the present study, the prevalence of *Hepatozoon* spp. was higher in adults (70.31%), then in young adults (55.50%) and finally in juveniles (50%), corroborating the sentence above. Since adults and young adults feed on intermediate hosts, such as frogs and fish. Furthermore, adults are susceptible to more years of exposure to the vector and parasite, compared to juveniles.

Moreover, the high prevalence of *Hepatozoon* in caiman populations from Brazil is usually reported. The prevalence of *Hepatozoon* spp. found in this study based on blood smears screening (70%) among *C. crocodilus* from Mato Grosso State was similar to *C. crocodilus yacare* (70%) from North-Pantanal, *C. crocodilus* (76%) in the Amazon region (Lainson, 1977), and *C. yacare* in western (71%) and southeastern (76-79%) Pantanal in Brazil (Bouer et al., 2017; Soares et al., 2017; Viana et al., 2010; Viana and Marques, 2005).

According to Viana et al. (2010), *H. caimani* infection can persist in the animal for a long time, more than six months of monitoring. In this study, all animals were collected, inspected for ectoparasites, measured, weighed, and identified (following the Brazilian identification standards for crocodilians imposed by IBAMA). Interestingly, in the present study, an adult male *C. crocodilus,* measuring 1.8 m, was collected (2017), and blood was extracted and released at the site of capture, one year later (2018), that same individual was randomly collected and blood was extracted. Both sampling events revealed the individual positive with *H. caimani*, supporting the observations of longevity made by Viana et al. (2010) although it is not possible to confirm if the infection persisted for the entire year, or if the species had been reinfected in the wild.

The variety of morphological and morphometric forms of blood developmental stages and tissue merogony observed in this study emphasizes the need and effectiveness of molecular confirmation and characterisation of hemogregarines revealing the presence of more than one species of *Hepatozoon* infecting caimans. In addition, some morphological characters that have never been described before for *Hepatozoon* in caimans were highlighted. In the literature, there are some reports of these morphological variations. According to Telford Junior et al. (2004), the plasticity of the blood developmental stages might be related to the host species involved. Other explanations were the adaptation of *Hepatozoon* species to different environments and hosts, or its low host specificity (Smith, 1996; Telford Junior et al., 2004). Besides, it was possible to observe significant intragenotipic variation among the isolates of *Hepatozoon* spp. in the present study, which could be linked to the morphological differences observed. Although amplification with the 18S rRNA gene revealed the separation of the genotypes of *Hepatozoon* spp. isolated from Brazilian caimans in the present study, more data on other genes (mitochondrial, for example) should be implemented to better understand this diversity of genotypes, and perhaps, identify as new species.

Therefore, This paper brings new insights into the gene and morphology diversity of *Hepatozoon* infecting *Caiman crocodilus* from Brazil.

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References

BOUER, A., ANDRÉ, M.R., GOLÇALVES, L.R., LUZZI, M.C., OLIVEIRA, J.P., RODRIGUES, A.C., VARANI, A.M., MIRANDA, V.F.O., PERLES, L., WERTHER, K. and MACHADO, R.Z., 2017. *Hepatozoon caimani* in *Caiman crocodilus yacare* (Crocodylia, Alligatoridae) from North Pantanal, Brazil. *Revista Brasileira de Parasitologia Veterinária*, vol. 26, no. 3, pp. 352-358. http://doi.org/10.1590/s1984-29612017041. PMid:28902260.

CLEMENTE, G.R.C., GUTERREZ-LIBERATO, G.A., ANJOS, C.C., SIMÕES, P.I., MUDERK, J.R., FECCHIO, A., LIMA, J.H.A., OLIVEIRA, P.M.A., PINHO, J.B., MATHIAS, B.S., GUIMARÃES, L.O. and KIRCHGATTER, K., 2023. Occurrence of *Hepatozoon* in Some Reptiles from Brazilian Biomes with Molecular and Morphological Characterization of *Hepatozoon caimani*. *Diversity (Basel)*, vol. 15, no. 12, pp. 1192. http://doi.org/10.3390/d15121192.

COOK, C.A., SMIT, N.J. and DAVIES, A.J., 2009. A redescription of *Haemogregarina fitzsimonsi* Dias, 1953 and some comments on *Haemogregarina parvula* Dias, 1953 (Adeleorina: Haemogregarinidae) from Southern African tortoises (Cyptodira: Testudinidae) with new host data and distribution records. *Folia Parasitologica*, vol. 56, no. 3, pp. 173-179. http://doi.org/10.14411/fp.2009.021. PMid:19827360.

DUSZYNSKI, D.W., MCALLISTER, C.T. and TELLEZ, M., 2020. The coccidian (Apicomplexa) of the Archosauria (Crocodylia: Eusuchia) of the world. *The Journal of Parasitology*, vol. 106, no. 1, pp. 90-122. http://doi.org/10.1645/19-73. PMid:31999218.

EISEN, R.J. and SCHALL, J.J., 2000. Life history of malaria parasite (*Plasmodium mexicanum*): independent traits and basis for variation. *Proceedings. Biological Sciences*, vol. 267, no. 1445, pp. 793-799. http://doi.org/10.1098/rspb.2000.1073. PMid:10819149.

GUINDON, S. and GASCUEL, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, vol. 52, no. 5, pp. 696-704. http://doi.org/10.1080/10635150390235520. PMid:14530136.

KEARSE, M., MOIR, R., WILSON, A., STONES-HAVAS, S., CHEUNG, M., STURROCK, S., BUXTON, S., COOPER, A., MARKOWITZ, S., DURAN, C., THIERER, T., ASHTON, B., MEINTJES, P. and DRUMMOND, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Journal of Bioinformatics*, vol. 28, no. 12, pp. 1647-1649. http://doi.org/10.1093/bioinformatics/bts199. PMid:22543367.

LAINSON, R., PAPERNA, I. and NAIFF, R.D., 2003. Development of *Hepatozoon caimani* (Carini, 1909) Pessôa, de Biasi e de Souza, 1972 in the caiman *Caiman c. crocodilus*, the frog *Rana catesbeiana* and the mosquito *Culex fatigans*. *Memorias do Instituto Oswaldo Cruz*, vol. 98, no. 1, pp. 103-113. http://doi.org/10.1590/S0074-02762003000100014. PMid:12700868.

MILLER, M.A., PFEIFFER, W. and SCHWARTZ, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Proceedings of the 2010 Gateway Computing Environments Workshop (GCE)*, 2010, New Orleans, LA, USA. USA: IEEE. pp. 1-8. http://doi.org/10.1109/GCE.2010.5676129.

NETHERLANDS, E.C., COOK, C.A., DU PREEZ, L.H., VANHOVE, M.P.M., BRENDONCK, L. and SMIT, N.J., 2018. Monophyly of the species of *Hepatozoon* (Adeleorina: Hepatozoidae) parasiting (African) anurans, with the description of three new species from hyperoliid frogs in South Africa. *Parasitology*, vol. 145, no. 8, pp. 1039-1050. http://doi.org/10.1017/S003118201700213X. PMid:29198245.

O'DWYER, L.H., MOÇO, T.C., PADUAN KDOS, S., SPENASSATTOM, C., SA SILVA, R.J. and RIBOLLA, P.E., 2013. Description of three new species of *Hepatozoon* (Apicomplexa, Hepatozoidae) from Rattlesnakes (*Crotalus durissus terrificus*) based on molecular, morphometric and morphologic characters. *Experimental Parasitology*, vol. 135, no. 2, pp. 200-207. http://doi.org/10.1016/j.exppara.2013.06.019. PMid:23867148.

PEREIRA, G.R., SOARES, P., HOMES, M.Q., VIANA, L.A., ABREU MANSO, P.P., MACHADO, M.P. and LOURENÇO-DE-OLIVEIRA, R., 2014. Are fish paratenic natural hosts of the caiman haemoparasite *Hepatozoon caimani*? *Parasitology Research*, vol. 113, no. 1, pp. 39-45. http://doi.org/10.1007/s00436-013-3623-9. PMid:24142284.

PERKINS, S.L. and KELLER, A.K., 2001. Phylogeny of nuclear small subunit rRNA genes of hemogregarines amplified with specific oligonucleotídeos. *The Journal of Parasitology*, vol. 87, no. 4, pp. 870-876. http://doi.org/10.1645/0022-3395(2001)087[0870:PONSSR]2.0.CO;2. PMid:11534653.

POSADA, D., 2003. Using MODELTEST and PAUP* to select a model of nucleotide substitution. *Current Protocols in Bioinformatics*, vol. 6, no. 1, pp. 6.5.1-6.5.14. http://doi.org/10.1002/0471250953.bi0605s00. PMid:18428705.

RAMBAUT, A., 2012 [viewed 20 November 2022]. *FigTree v1.4. Molecular evolution, phylogenetics and epidemiology* [online]. Edinburgh: University of Edinburgh, Institute of Evolutionary Biology. Available from: http://tree.bio.ed.ac.uk/software/figtree/

SANTOS, S.A., STOLL, M.N., PINHEIRO, M.S., CAMPOS, Z., MAGNUSSON, W.E. and MOURÃO, G., 2011 [viewed 20 November 2022]. Diets of *Caiman crocodilus yacare* from diferente habitats in Brazilian Pantanal. *The Herpetological Journal* [online], vol. 6, pp. 111-117. Available from: https://repositorio.inpa.gov.br/handle/1/19380

SEBBEN, A. 2007. Microdissecaçao fisiologica a fresco: uma nova visão sobre a anatomia de anfíbios e répteis, In: L.B. NASCIMENTO, M.E. OLIVEIRA eds. *Herpetologia no Brasil II*. Belo Horizonte: Sociedade Brasileira de Herpetologia, pp. 311-325.

SMITH, T.G., 1996. The genus *Hepatozoon* (Apicomplexa: adeleina). *The Journal of Parasitology*, vol. 82, no. 4, pp. 565-585. http://doi.org/10.2307/3283781. PMid:8691364.

SOARES, P., BORGHESAN, T.C., TAVARES, L.E.R., FERREIRA, V.L., TEIXEIRA, M.M.G. and PAIVA, F., 2017. *Hepatozoon caimani* Carini 1909 (Adeleina: Hepatozoidae) in wild population of *Caiman yacare* Daudin, 1801 (Crocodylia: Alligatoridae), Pantanal, Brazil. *Parasitology Research*, vol. 116, no. 7, pp. 1907-1916. http://doi.org/10.1007/s00436-017-5467-1. PMid:28512673.

TELFORD JUNIOR, S.R., 1984. Haemoparasites of reptiles. In: G.L. HUFF, F.L. FRYE and E.R. JACOBSON, eds, *Diseases of 506*

amphibians and reptiles. New York: Plenum Press, pp. 385-517. http://doi.org/10.1007/978-1-4615-9391-1_20.

TELFORD JUNIOR, S.R., 2009. *Hemoparasite of the reptilian: color atlas and text*. Boca Raton: CRC Press.

TELFORD JUNIOR, S.R., ERNST, J.A., CLARCK, A.M. and BUTLER, J.F., 2004. *Hepatozoon sauritus*: a polytopic hemogregarine of three genera and four species of snakes in northern Florida, with specific identity verified from genome analysis. *The Journal of Parasitology*, vol. 90, no. 2, pp. 352-358. http://doi.org/10.1645/GE-3258. PMid:15165059.

TELLEZ, M., 2013. *A checklist of host parasite interactions of the order Crocodylia*. Berkeley: University of California Press. http://doi.org/10.1525/california/9780520098893.001.0001.

UETANABARO, M., 1989. *Hábito alimentar de Caiman crocodilus yacare (Crocodilia, Alligatoridae) no Pantanal do Sul Mato-grossense*. Rio Claro: UNESP, 79 p. Dissertação de Mestrado.

UJVARI, B. and MARQUES, E.J., 2005. High prevalence of *Hepatozoon* spp. (Apicomplexa: Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical Australia. *The Journal of Parasitology*, vol. 90, no. 3, pp. 670-672. http://doi.org/10.1645/GE-204R. PMid:15270125.

VELASCO, A., COLIMINE, G., SOLA, R. and VILLARROEL, G., 2009. Effects of sustained harvests on wild populations of *Caiman crocodilus crocodilus* in Venezuela. *Interciencia*, vol. 28, pp. 544-548.

VIANA, L.A. and MARQUES, E.J., 2005. Haemogregarine parasites (Apicomplexa: Hepatozoidae) in *Caiman crocodilus yacare* (Crocodilia: Alligatoridae) from Pantanal, Corumba, MS, Brazil. *Revista Brasileira de Parasitologia Veterinária*, vol. 14, no. 4, pp. 173-175. PMid:16445875.

VIANA, L.A., PAIVA, F., COUTINHO, M.E. and LOURENÇO-DE-OLIVEIRA, R., 2010. *Hepatozoon caimani* (Apicomplexa: Hepatozoidae) in wild caiman, *Caiman yacare*, from the Pantanal Region, Brazil. *The Journal of Parasitology*, vol. 96, no. 1, pp. 83-88. http://doi.org/10.1645/GE-2150.1. PMid:19685936.

ZIPPEL, K.C., LILLYWHITE, H.B. and MLADINICH, C.R., 2001. New vascular system in reptiles: anatomy and postural hemodynamics of the vertebral venous plexus in snakes. *Journal of Morphology*, vol. 250, no. 2, pp. 173-184. http://doi.org/10.1002/jmor.1063. PMid:11746458.