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Hemogregarine Diversity Infecting Brazilian Turtles with a Description of Six New Species of *Haemogregarina* (Apicomplexa: Adeleorina: Haemogregarinidae)

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Abstract: (1) Background: Hemoparasites of the genus *Haemogregarina* (Haemogregarinidae) are commonly reported in freshwater turtles. However, in Brazil, only three species have currently been characterised using molecular methods. This study aimed to bring new insights on the diversity of species of *Haemogregarina* infecting Brazilian freshwater turtles from Mato Grosso and Goiás states using molecular and morphological tools. (2) Methods: In total, 2 mL of blood was collected, with between two to five blood smears prepared, fixed with absolute methanol, and stained with a 10% Giemsa solution. Blood was stored at −20 °C for molecular analysis targeting the 18S rRNA gene. Fragments of the organs (liver, spleen, heart, and kidney) were separated and stained with hematoxylin-eosin. (3) Results: A total of 40 turtles were screened and hemogregarines were observed in 34 (85%) individuals (3 *Podocnemis expansa* and 31 *Podocnemis unifilis*). *Haemogregarina embaubali* and six new species of *Haemogregarina* were observed and formally described as follows: *H. unifila* n. sp., *H. rubra* n. sp., *H. goianensis* n. sp., *H. araguaiensis* n. sp., *H. tigrina* n. sp., and *H. brasiliana* n. sp. (4) Conclusions: This study contributes to the diversity and knowledge of Brazilian fresh-water turtle blood parasites, using integrative approaches for diagnosing and characterizing hemoparasites, with the identification of six undescribed species.

Keywords: Hemoparasite; PCR; taxonomy; fresh-water turtles; Brazil; diversity

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1. Introduction

Wild animals are exposed to a variety of pathogens and parasites, such as hemoparasites. The presence of these organisms may cause disorders, such as hemolytic anemia [1]. The most common hemoparasite found in chelonians (Reptilia: Testudines) is an obligatory intracellular parasite of the Phylum Apicomplexa (Levine, 1970) and Suborder Adeleorina Léger, 1911, named the haemogregarine group, that is constituted by four families: Dactylosomatidae (Jakowska and Nigrelli, 1955), Karyolysidae Labbé, 1894, Hepatozoidae Wenyon, 1926, and Haemogregarinidae Léger, 1911. Tortoises and freshwater turtles are reported to be infected with species of *Hemolivia* Petit, Landau, Baccam & Lainson 1990 (Karyolysidae) [2–4]; land tortoises and semi-aquatic turtles with species of *Hepatozoon* Miller, 1908 (Hepatozoidae) [5,6]; and freshwater turtles can be infected with species of *Haemogregarina* Danilewsky, 1885 (Haemogregarinidae) [7–18].

Haemogregarina spp. (Apicomplexa: Adeleina: Haemogregarinidae) follow a heteroxenous lifecycle [19], with intermediate vertebrate hosts such as fish and turtles, and definitive invertebrate vectors such as gnathiid isopods and leeches of the families Glossiphonidae, Ozobranchidae, and Pisicolidae [20–23]. In turtles, *Haemogregarina balli* has the best-described lifecycle. This parasite is reported from the fresh-water turtle, *Chelydra serpentina*, and the leech *Placobdella ornata* as its vector [24]. Transmission occurs through

blood hematophagy by leeches; the merozoites present in the salivary ductules of the proboscis are released into the turtle during feeding. In the vertebrate host, the parasite develops in organs, such as the lungs, spleen, and liver; and can be found circulating in the peripheral blood along with intraerythrocytic developmental stages, such as merozoites, pre-meronts, meronts, and immature and mature gamonts [8,24–26].

In Brazil, several studies on the prevalence, morphology, and molecular characterisation of *Haemogregarina* spp. infecting vertebrate hosts have been completed [14,16,18,27]. Moreover, studies on Brazilian chelonid haemogregarines have been restricted to only five host species of the 36 that occur in the country [28]. Namely, *Phrynops geoffroanus* (Schweigger, 1812) [29], *Podocnemis expansa* (Schweigger, 1812) [14,30,31], *Podocnemis unifilis* (Troschel, 1848) [16,27], *Podocnemis sextuberculata* Cornalia, 1849 [18], and *Mesoclemmys vanderhaegei* (Bour, 1973) [32].

This study aimed to increase the diversity of Brazilian freshwater turtle blood parasites and demonstrate the importance of using integrative approaches for the diagnosis and characterisation of hemoparasites, with the identification of six undescribed species.

2. Materials and Methods

2.1. Site and Animal Collection

The study was submitted and authorized by the Ethics Committee on Animal Use of the Institute of Biosciences, Universidade Estadual Paulista "Júlio de Mesquita Filho", (CEUA–UNESP, protocol number 1061), and by the Authorization and Information System in Biodiversity (SISBIO, process no. 61940). Fieldwork was carried out from 2017 to 2020, as reported in Table 1.

Table 1. Data on localities, municipalities, years, states, and coordinates where the testudines were collected.

Locality	Municipality	Year	State *	Coordinates
São Sebastião do Paraíso farm	Boa Esperança do Sul	2019	SP	21°51′36.83″ S, 48°27′3.56″ W
Vermelho river	Britânia	2017	GO	15°10′44.82″ S, 51°9′58.48″ W
Araguaia river	Itacaiú	2019	GO	15°04′39.81″ S, 51°26′68,91″ W
Stream (unnamed)	Nova Xavantina	2018/2020	MT	14°31′48.03″ S, 51°41′43.88″ W
Sertaneja Retiro farm	Cocalinho	2018/2020	MT	14°26′19.98″ S, 51°35′20.17″ W
Pindaíba river	Araguaiana	2018/2020	MT	14°35′47″ S, 51°43′9.59″ W

* São Paulo state (SP), Goiás state (GO), Mato Grosso state (MT).

A total of 40 chelonids were screened from haemogregarines; namely, four *Chelonoidis carbonarius* (Spix, 1824), two *Phrynops geoffroanus* (Schweigger, 1812), three *Podocnemis expansa* (Schweigger, 1812), and 31 *Podocnemis unifilis* Troschel, 1848. For the blood collection, the animals were physically restrained with appropriate equipment following adequate norms [33]. The free-living aquatic turtles were captured by fishing with barbless fishing hooks [33]; in addition, the free-living terrestrial turtles were captured manually by the carapace and immobilized by the physical containment captured [14], and tagged by carapacial perforation [34]. During the sampling period, sex (male/female) was determined based on sexual dimorphism, and the age (adult/juvenile/new hatchling) of the turtles was estimated as described by Araújo et al. [35].

Approximately 2 mL of blood was collected by cervical paravertebral venous puncture [36]. This sample was divided into two aliquots. One was used to make two to five blood smears at the collection site. The remaining volume was placed in a polystyrene tube containing EDTA as an anticoagulant and stored at −20 °C until the samples could

be molecularly analyzed in the Sector of Parasitology of the Institute of Biosciences at São Paulo State University "Julio de Mesquita Filho", UNESP–IBB.

2.2. Blood Smears, Histological Slides, and Morphological Data

Blood smears were fixed with absolute methanol for 5 min and stained with 10% Giemsa for 1 h [37]. For morphological analysis of the intraerythrocytic parasite stages, digital images were captured and measured using a compound microscope at 1000× magnification with the Leica software application suite LAS V3.8 (Leica Microsystems). Measurements are in micrometres (µm), comprising the parasite's length, width, and area (μm^2), with mean and standard deviation (means ± standard deviation) given.

The morphology of haemogregarine developmental stages was analyzed based on previous descriptions [8,12,13,16,18,27]. Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear [38].

For histopathological analysis, the turtles were euthanized using 50 mg/kg Tiopentax®, a commercial anaesthetic administered intracerebrally [39], according to the Animal Ethics Committee of Veterinary Medicine. The histological slides of the liver, spleen, heart, and kidney were sectioned longitudinally and transversally (the measurements can vary according to the position of the histological cut), fixed in 4% buffered neutral formalin, and stained with hematoxylin-eosin [37].

2.3. Molecular Data

DNA was extracted from whole blood and tissue samples following the blood protocol of the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Two PCR assays were performed targeting two different regions of the 18S rDNA of apicomplexan parasites using the HepF300 and Hep900 pair of primers, which amplifies 600 bp [40]; and the Hemo1 and Hemo2 pair of primers, which amplifies 900 bp [41].

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 MyFi™ 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) following the conditions of O'Dwyer et al. [42]. 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 3500 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 [43].

For each positive animal, the newly generated sequences, amplified with the pair of primers HepF300/Hep900 and Hemo1/Hemo2, were compared with other isolates from haemogregarine parasites available on GenBank. The sequences of partial 18S rDNA were aligned using Geneious version 7.1.3 [43], with the MUSCLE algorithm implemented from within Geneious version 7.1.3 (Biomatters, www.geneious.com) and default settings with related sequences that appeared on Blastn search. The phylogenetic analyses were performed using both alignments using the Bayesian inference (BI) and maximum likelihood (ML) methods. JModelTest v.2.1.10 [44] was used for the ML method to identify the best evolutionary model. Based on the Akaike information criterion (AIC), the transitional model [45] with a discrete Gamma distribution (TVM + G) was chosen. Phylogenetic analysis was inferred using PhyML [46] with 1000 replicate bootstraps (>50%).

The BI analysis was carried out using MrBayes implemented from the computational resource CIPRES [47]; the best BIC score was the general time reversible model (GTR + I + Γ) [48]. The Markov chain Monte Carlo (MCMC) algorithm was run for 10,000,000 generations, sampling one tree every 1000 generations. For burn-in, the first 25% of generations were

discarded, and the consensus tree was estimated using the remaining trees. The Bayesian posterior probabilities (BPP) cut-off considered was >50%.

Two phylogenetic analyses were performed; one with isolates from adeleorinid parasites (Haemogregarinidae, Hepatozoidae, Karyolysidae, and Dactylosomatidae) available from GenBank, which were used to construct both trees (BI and ML). The phylogenetic trees for both BI and ML analyses were edited in FigTree v1.4 [49]. *Adelina dimidiata* (DQ096835) and *Adelina grylli* (DQ09686) were selected as outgroups. For the second analysis, the BI and ML trees were performed only with *Haemogregarina* isolates available on GenBank from turtles and fish. *Hepatozoon simidi* (MT754271) and *Hepatozoon fitszimonsi* (KR069084) were selected as outgroups. A pair-wise distance (p-distance) matrix was used to compare the interspecific divergence between species of *Haemogregarina* sequences isolated from chelonids.

3. Results

3.1. Prevalence

Through morphological analysis of the blood smears from 40 chelonids screened, haemogregarine parasites were observed in 34 (85%) specimens: 3 *P. expansa* and 34 *P. unifilis* (Table 2).

Table 2. Data on Brazilian testudines positives for *Haemogregarina* species through morphological analysis collected in 2017–2020 from Mato Grosso, Goiás, and São Paulo States.

Testudines: Pelurodira	Family	Year	State	N	P
Chelonoidis carbonarius (Spix, 1824)	Testudinidae	2018/2020	MT	4	0
Phrynops geoffroanus (Schweigger, 1812)	Chelidae	2019	GO, SP	2	0
Podocnemis expansa (Schweigger, 1812)	Podocnemididae	2017/2019	GO	3	3
Podocnemis unifilis (Troschel, 1848)	Podocnemididae	2017/2019	GO, MT	31	31

MT (Mato Grosso State). GO (Goiás State). SP (São Paulo State). N (negative hosts). P (positive hosts).

3.2. Molecular Data

The samples diagnosed as positive by conventional microscopy were successfully amplified and sequences targeting 18S rRNA gene were generated. Each sequence amplified with Hep300/Hep900 (~600) was compared with the others from this study and with the sequences available on GenBank; the same was made with Hemo1/Hemo2 (~900) (Table 3).

Through gene similarity comparison, four genotypes of *Haemogregarina embaubali* Correa, Picelli, da Silva, Valadão, Hernández-Ruz, and Viana, 2022 were revealed. The isolates amplified with the HepF300 and Hep 900 primer pair revealed: the first genotype comprising 12 identical isolates shared 100% similarity to the isolates of the original description by Correa et al. (2022); the second genotype comprising six identical isolates had 99.8% gene similarity with the original description; the third genotype comprising one isolate had 99.4% gene similarity with the isolates from the original description; and the fourth genotype comprising one isolate had 99% similarity with the isolates from the original description. In addition, six undescribed species were observed: *Haemogregarina unifila* n. sp. in five *P. unifilis* specimens; *Haemogregarina rubra* n. sp. in one *P. unifilis* specimen; *Haemogregarina goianensis* n. sp. in two specimens, one *P. unifilis*, and one *P. expansa*; *Haemogregarina araguaiensis* n. sp. in two *P. unifilis* specimens; *Haemogregarina tigrina* n. sp. in two *P. unifilis* specimens; and *Haemogregarina brasiliana* n. sp. in two *P. unifilis* specimens. Complete data on these new species can be found in the species description section below. Similar results were observed regarding the amplification using the Hemo 1 and Hemo 2 primer pair (Table 3).

Table 3. Gene similarity (%) of *Haemogregarina* isolates targeting the 18S gene with pair of primers HepF300 and Hep900 (down side) and with pair of primers Hemo 1 and Hemo 2 (upper side).

***Haemogregarina* Isolates**	**1.**	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**
1. OQ377557 - *H. rubra* n. sp.		98.1	98.9	--------	98.1	98.1	98.3	97.2	98.5	98.3
2. OQ388265 - *H. araguaiensis* n. sp.	96.33		97.7	--------	97.5	97.5	97.8	96.9	97.3	97.9
3. OQ377555 - *H. goianensis* n. sp.	95.95	99.61		--------	97.5	97.8	97.8	96.9	97.1	97.4
4. OQ377710 - *H. unifila* n. sp.	93.82	96.52	96.32		--------	--------	--------	--------	--------	--------
5. OQ377129 - *H. brasiliana* n. sp.	94.02	96.91	96.71	98.84		99.7	98.6	96.9	99.3	99.4
6. OQ377558 - *H. tigrina* n. sp.	94.40	97.29	97.10	98.45	99.61		98.5	97.0	99.7	99.7
7. OQ377547 - *H. embaubali* (genotype 4)	93.24	95.55	95.36	98.84	98.45	98.26		97.2	98.5	98.6
8. OQ377462 - *H. embaubali* (genotype 3)	92.86	95.55	95.36	99.03	98.26	97.87	99.03		96.7	97.0
9. OQ377458 - *H. embaubali* (genotype 2)	93.44	96.13	95.94	99.61	98.84	98.45	99.23	99.42		99.81
10. OQ377457 - *H. embaubali* (genotype 1)	93.24	95.94	95.74	99.42	98.65	98.26	99.42	99.61	99.81	
11. MW540607—*H. embaubali*	93.24	95.94	95.74	99.42	98.65	98.26	99.42	99.61	95.81	100
12. MW540605—*H. karaja*	95.95	97.49	97.10	95.94	95.16	95.55	95.16	95.16	95.55	95.55
13. MF476203—*H. podocnemis*	92.47	95.16	95.35	98.26	97.49	97.29	98.26	98.45	98.65	98.84
14. HQ224959—*H. balli*	90.15	92.26	92.46	92.84	92.07	91.88	92.26	92.46	92.84	92.84
15. KM887507—*H. sacaliae*	89.77	92.26	92.46	93.23	92.46	92.26	92.65	92.84	93.23	93.23
16. KF257928—*H. stepanowi*	90.35	92.07	92.26	92.65	91.88	91.68	92.07	92.26	92.65	92.65
17. KM887509—*H. pellegrini*	90.35	92.28	92.47	92.86	92.08	91.89	92.28	92.47	92.86	92.86

Two phylogenetic analyses were performed by ML and BI methods. The first (Figure 1) comprised various haemogregarine species isolates (Dactylosomatidae, Haemogregarinidae, Hepatozoidae, and Dactylosoma), revealing a paraphyly in regards to *Haemogregarina* isolates, with two well-supported clades observed. The first clade was recovered as a sister group to isolates of the Hepatozoidae and Karyolysidae clades, and the second *Haemogregarina* clade was recovered as a sister group to isolates of the Dactylosomatidae. The isolates from the present study were located in a well-supported clade comprising isolates from the Neotropical region. *Haemogregarina karaja*, *H. rubra* n. sp., *H. goianensis* n. sp., and *H. araguaiensis* n. sp. formed a well-supported monophyly sister to a large monophyletic clade comprising *H. podocnemis*, *H. embaubali*, *H. unifila* n. sp., *H. brasiliana* n. sp., and *H. tigrina* n. sp. The isolates of *H. embaubali* formed a polytomy with *H. podocnemis*, sister to a clade comprising *Haemogregarina brasiliana* n. sp. and *Haemogregarina tigrina* n. sp.

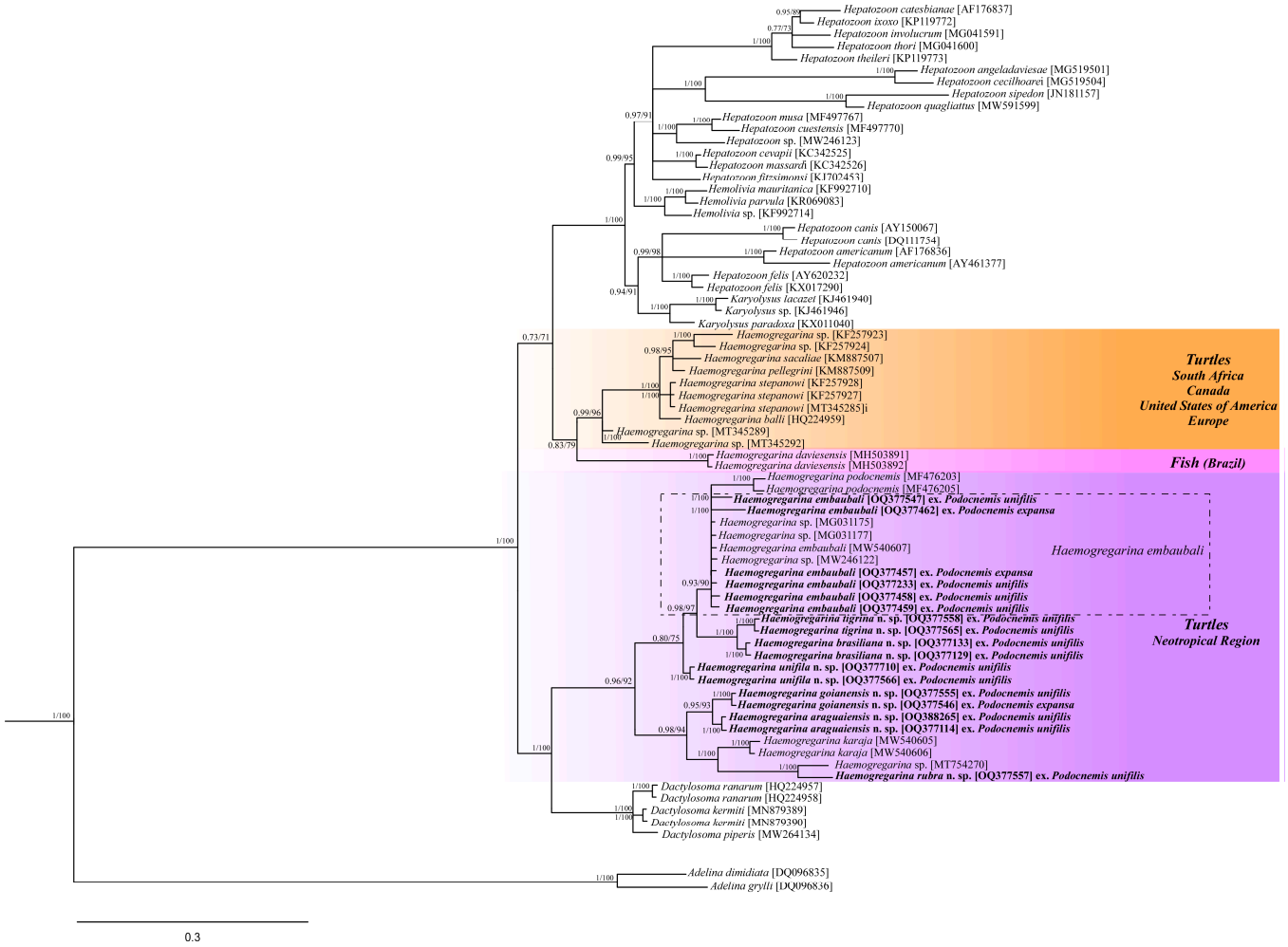

Figure 1. Consensus phylogram of haemogregarines based on 18S rDNA sequences. (587 bp). The topology trees were inferred by Bayesian (BI) and maximum likelihood (ML) methods (represented by BI tree). The isolates *Adelina dimidiata* (DQ096835) and *Adelina grylli* (DQ09686) were used as an out-group.

The second phylogenetic analysis (Figure 2), constituted only by *Haemogregarina* isolates from turtles and fish, revealed three well-supported clades. The first (I) comprised isolates from Neotropical turtle hosts from Brazil and Colombia. The second (II) comprised isolates from turtles and fish around the world, including Brazil; and the third (III) included isolates from turtles from Bulgaria, Canada, Asia, and Africa. The isolates from the present study grouped in the first clade, with other *Haemogregarina* isolates from

Brazilian freshwater turtles. As in Figure 1, the isolates of *H.embaubali* also formed a polytomy with *H. podocnemis*, sister to a clade comprising *Haemogregarina brasiliana* n. sp. and *Haemogregarina tigrina* n. sp.

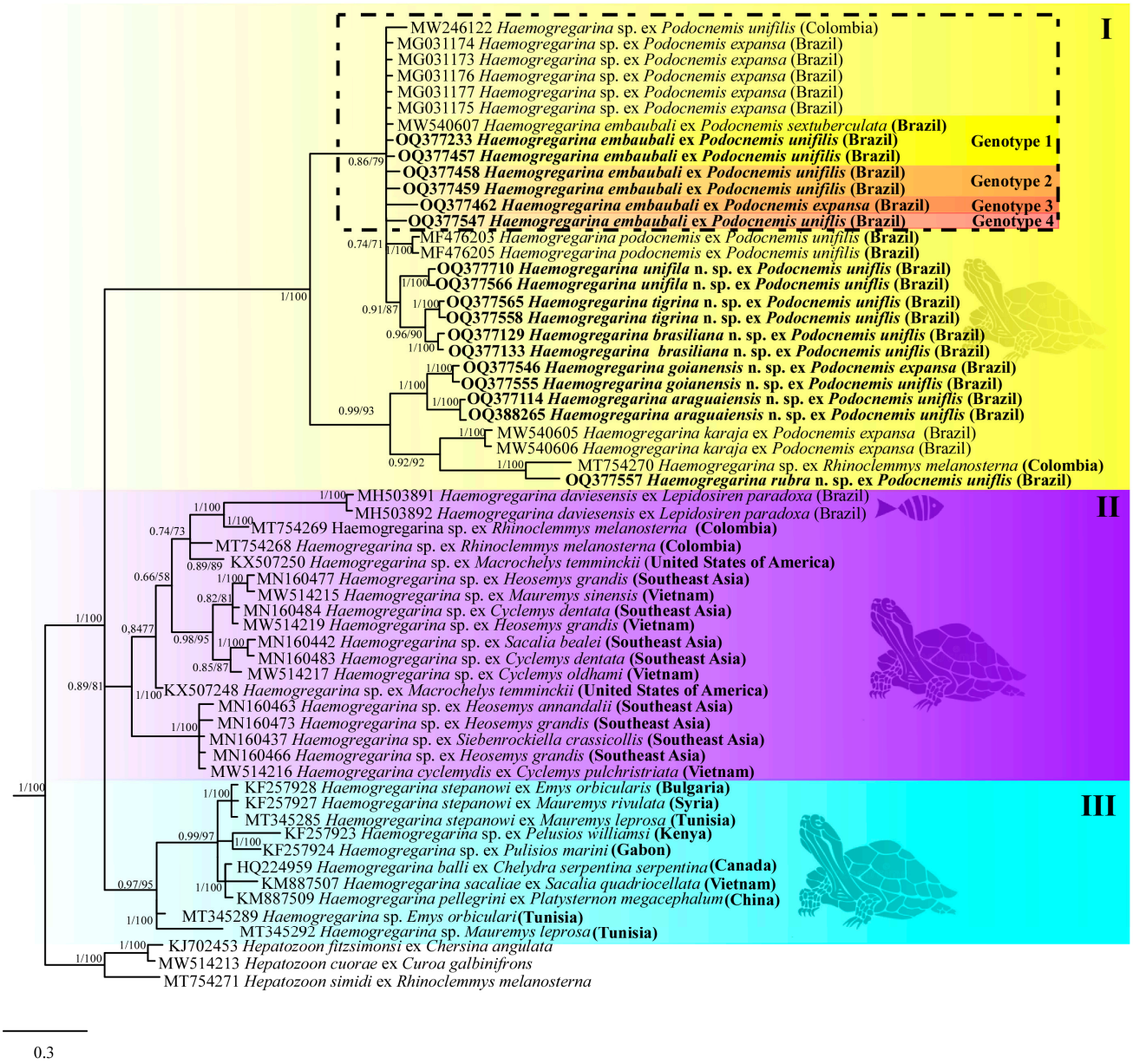

Figure 2. Consensus phylogram of *Haemogregarina* spp. from turtles and fish based on 18S rDNA sequences (539 bp). The topology trees were inferred by Bayesian (BI) and maximum likelihood (ML) methods (represented by BI tree). The isolates *Hepatozoon fitzsimonsi* (KJ702453), *Hepatozoon cuorae* (MW514213), and *Hepatozoon simidi* (MT754271) from turtles were used as an out-group.

3.3. Morphological Data

Using compound microscopy for the morphological and morphometric analyses, it was possible to observe merozoites, trophozoites, gamonts with cytoplasmic vacuoles, immature gamonts, mature gamonts, pre-meronts, and meronts in the circulating blood. In addition, tissue merogony was observed and characterised in the fresh-water turtles' liver and spleen. The results correspond to the molecular data, with different morphologies and morphometries observed.

The first species identified was *Haemogregarina embaubali*, recently described by Correa et al. [18]. This species was found parasitising in 20 freshwater turtles, two *P. expansa* and 18 *P. unifilis* (Table 4; Figure 3). Trophozoites, gamonts with vacuoles, pre-meronts, meronts, immature gamonts, and two mature gamonts were observed. The trophozoites are small and shaped like "comma", with the nuclei staining dark-purple and occupying half of the parasite's body, and displaced to the rounded side of the parasite. The other side of the parasite is tapered; and the cytoplasm contains vacuoles, staining bluish in color. The gamonts contain larger vacuoles, with both ends rounded and cytoplasm stained bluish. The gamont's nuclear chromatin is concentrated on the round side of the parasite's body. The pre-meronts possess a similar shape to the gamonts containing vacuoles; however, in most cases, the nuclear chromatin is loosely arranged throughout the cytoplasm, with cytoplasmic vacuoles also visible. The meronts are larger, with nuclear division evident and staining dark purple. Both ends of the parasite's meronts are rounded. Regarding the smaller mature gamont, the cytoplasm is stained bluish. In some cases, cytoplasmic vacuoles and granules were observed. The nuclei are displaced to the larger and rounded side of the parasite, staining purple. Sometimes, it is possible to observe parasitophorous capsules, displacement of the nuclei, and cell hypertrophy. The larger mature gamonts cytoplasm stain purple with the nuclei staining pinkish-purple. Both ends are rounded containing a parasitophorous capsule. The nuclei extend the width of the parasite, dividing the cytoplasm in two, with one side cytoplasm displaying granules and the other side, clear. Due to the parasite's size, the host cell undergoes hypertrophy and distortion, sometimes even rupturing.

Table 4. *Haemogregarina* species measurements (mean ± standard deviation) in micrometers (μm and μm^2) of Brazilian fresh-water turtle's blood and tissue stages.

Haemogregarina	DS	N	P (%)	C	PL	PW	PA	NL	NW	NA
	T	4	0.99		7.26 ± 0.34	3.50 ± 0.19	19.38 ± 3.44	2.63 ± 0.81	3.50 ± 0.19	
	IG	5			11.3 ± 0.12	5.95 ± 0.37	55.0 ± 1.29	3.44 ± 0.14	3.99 ± 0.25	
***H. rubra* n. sp.**	PM	2			11.7 ± 0.08	5.31 ± 0.44	56.2 ± 2.02			
	MG1	5			16.98 ± 0.92	7.55 ± 0.79	113.79 ± 9.47		4.26 ± 0.60	
	MG2	8			20.08 ± 0.61	9.56 ± 0.76	164.27 ± 6.33	6.13 ± 1.08	7.82 ± 1.69	
	T	7	1.4 and 2.14		10.53 ± 1.66	3.40 ± 0.28	28.66 ± 1.30	3.06 ± 0.04	2.77 ± 0.27	5.82 ± 0.68
	PM	5			12.57 ± 0.45	3.75 ± 0.78	30.26 ± 2.08	3.12 ± 0.97	2.44 ± 1.87	4.89 ± 1.99
	IG	15			10.72 ± 0.41	5.42 ± 0.82	42.82 ± 2.78	2.67 ± 0.62	1.20 ± 0.19	4,44 ± 0.53
	MG1	20			13.65 ± 1.04	6.11 ± 0.26	62.69 ± 5.14	4.76 ± 1.47	4.35 ± 0.99	10.30 ± 1.89
	MG2	20			21.01 ± 4.99	11.50 ± 2.78	180.0 ± 21.08	6.99 ± 2.65	7.76 ± 2.56	30.05 ± 11.02
	T	5	0.6 and 5.0		8.49 ± 0.62	3.84 ± 0.63	25.52 ± 3.70	3.51 ± 0.67	3.50 ± 0.19	9.82 ± 2.17
	PM	5			11.93 ± 1.42	7.08 ± 1.35	65.62 ± 18.14	7.09 ± 0.59	6.90 ± 0.91	38.8 ± 4.27
***H. goianensis* n. sp.**	IG	15			11.05 ± 2.64	4.58 ± 1.15	40.99 ± 9.23			
	MG1	20			11.57 ± 0.21	5.41 ± 0.34	56.24 ± 0.78	4.48 ± 0.12	0.80 ± 0.02	7.99 ± 2.01
	MG2	25			21.96 ± 2.07	9.28 ± 1.92	178.48 ± 40.10	7.85 ± 1.41	7.78 ± 1.03	30.77 ± 9.01
	T	5	1.0–6.42		7.03 ± 0.48	3.05 ± 0.33	16.04 ± 2.20	4.66 ± 0.08	2.57 ± 0.01	9.43 ± 0.96
	IG	15			7.99 ± 2.58	3.79 ± 1.44	26.74 ± 13.72	4.35 ± 1.01	3.95 ± 1.33	13.95 ± 5.52
	MG1	15			13.58 ± 0.06	6.13 ± 0.06	66.35 ± 2.05			
	MG2	15			19.43 ± 4.84	9.28± 1.46	150.65 ± 29.24	2.63 ± 1.37	3.47 ± 0.28	9.70 ± 0.12
	PM	3			10.55 ± 0.82	6.24 ± 1.99	61.74 ± 4.78	5.76 ± 0.35	7.35 ± 0.59	39.39 ± 11.50
	M	2			14.67 ± 0.78	8.25 ± 1.43	91.7 ± 3.24	4.46 ± 0.15	3.79 ± 0.06	11.04 ± 1.40
	PM	6	0.20 and 2.5		13.83 ± 2.22	6.25 ± 0.97	65.41 ± 10.12			
***H. brasiliana* n. sp.**	IG	15			12.87 ± 1.33	6.54 ± 1.76	62.78 ± 8.04	3.27 ± 1.17	1.06 ± 0.88	4.11 ± 0.58
	MG	10			21.38 ± 1.48	9.58 ± 0.77	168.16 ± 19.55	5.51 ± 0.52	7.14 ± 0.49	28.45 ± 1.61
	Me	5	3.9 and 5.0		6.55 ± 0.21	2.10 ± 0.33	14.07 ± 1.38	4.47 ± 1.30	1.99 ± 0.28	10.04 ± 1.85
	T	10			7.41 ± 0.78	3.03 ± 0.46	18.86 ± 2.13	4.28 ± 0.81	3.86 ± 0.47	13.12 ± 1.22
	PM	5			10.45 ± 2.92	5.12 ± 1.39	45.14 ± 15.07			
	M	3			21.57 ± 2.01	9.88 ± 1.78	174.32 ± 21.06			
	IG	25			11.02 ± 0.34	5.45 ± 1.40	45.14 ± 3.98	2.33 ± 0.88	0.98 ± 0.01	4.03 ± 0.87
	MG	15			25,37 ± 1.43	12.23 ± 0.78	230.01 ± 12.85	6.02 ± 0.34	9.40 ± 0.97	53.36 ± 7.35

Table 4. *Cont.*

Haemogregarina	DS	N	P (%)	C	PL	PW	PA	NL	NW	NA
	T	15	0.01–12.49		7.39 ± 1.44	3.24 ± 0.79	19.02 ± 1.64	3.80 ± 0.61	2.89 ± 0.31	6.10 ± 1.22
	GV	15			11.1 ± 0.78	4.3 ± 0.88	57.80 ± 5.01	5.0 ± 0.74	3.22 ± 0.95	11.08 ± 3.41
	PM	15			10.27 ± 0.60	5.18 ± 0.49	53.13 ± 6.01			
H. embaubali	M	5			12.50 ± 0.26	7.08 ± 0.46	85.27 ± 6.08	2.27 ± 0.25	1.00 ± 0.17	4.05 ± 0.55
(Genotype 1)	IG	25			10.30 ± 0.65	4.59 ± 0.73	50.84 ± 4.00	2.13 ± 0.21	2.98 ± 0.47	5.17 ± 1.33
	MG1	25			12.90 ± 1.27	6.85 ± 2.20	81.17 ± 3.38	3.19 ± 0.88	1.01 ± 0.12	5.06 ± 1.11
	MG2	30			20.10 ± 1.62	9.59 ± 0.83	134.82 ± 15.00	5.94 ± 1.89	7.26 ± 2.16	33.59 ± 4.97
	Me	10		3.75 ± 0.25	20.10 ± 1.62	13.21 ± 3.51	152.46 ± 77.36			
	GV	20			10.06 ± 0.79	5.80 ± 1.01	50.84 ± 4.45			
H. embaubali	GM	10	0.45–7.65		17.32 ± 1.14	7.88 ± 0.90	119.22 ± 3.56			
(Genotype 2)	PM	10			11.75 ± 1.31	6.33 ± 0.87	55.60 ± 2.64			
	T	15	13.97		5.88 ± 0.97	2.21 ± 0.43	16.46 ± 2.81	3.12 ± 0.39	2.18 ± 0.26	
	GV	15			8.13 ± 1.41	3.99 ± 0.41	30.3 ± 2.47			
H. embaubali	IG	15			7.98 ± 1.21	4.27 ± 0.54	33.72 ± 3.69	4.15 ± 0.15	2.12 ± 0.36	
(Genotype 3)	MG	20			10.42 ± 1.07	5.46 ± 0.70	49.12 ± 4.45	4.27 ± 0.20	2.19 ± 0.41	
	Me	10		3.03 ± 0.32	15.57 ± 2.51	12.66 ± 1.70	133.61 ± 22.99			
H. embaubali	IG	10	1.25		14.78 ± 0.21	7.00 ± 0.15	86.06 ± 1.47	3.66 ± 0.52	3.43 ± 0.22	9.11 ± 1.78
	MG	5			10.48 ± 3.22	5.56 ± 1.71	52.98 ± 16.81	3.85 ± 0.65	3.69 ± 0.45	10.82 ± 1.72
	PM	4			12.08 ± 0.96	6.29 ± 0.44	62.30 ± 6.86			

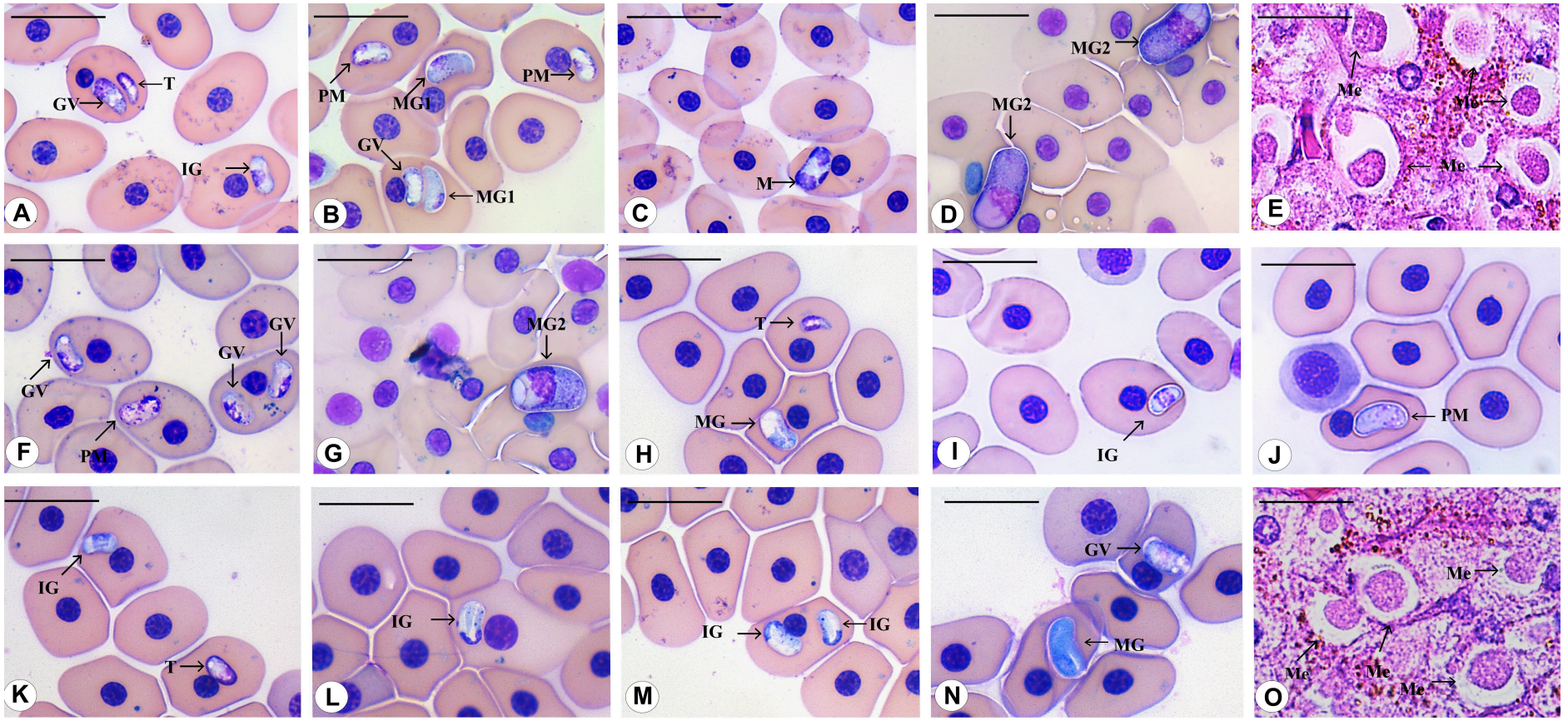

Figure 3. (**A**–**O**): Presence of different developmental stages and morphologies of *Haemogregarina embaubali*. In the blood smears (**A**–**N**) and liver histological slide (**E**,**O**) of *P. unifilis* and *P. expansa* collected from Mato Grosso and Goiás States, in 2017 to 2019. Scale: 20 µm. (**A**–**E**) *Haemogregarina embaulabi* genotype 1 observed in eleven *P. unifilis* and one *P. expansa*. (**F**,**G**) *Haemogregarina embaubali* genotype 2 observed in six *P. unifilis*. (**H**–**J**) *Haemogregarina embaulabi* genotype 3 observed in one *P. unifilis*. (**K**–**O**) *Haemogregarina embaulabi* genotype 4 observed in one *P. expansa*. T: trophozoite; IG: immature gamont; GV: gamont with vacuole; MG1: mature gamont 1; MG2: mature gamont 2; MG: mature gamont; PM: pre-meront; M: blood meront; Me: tissue meront (liver).

3.4. Species Descriptions

Taxonomic Summary
Phylum Apicomplexa Levine 1970
Class Coccidia Leuckart 1879
Order Eucoccidiorida Léger and Duboscq 1910
Suborder Adeleorina Léger 1911
Family Haemogregarinidae (Neveu-Lemaire) Léger 1911
Genus *Haemogregarina* Danilewsky 1885
Diagnosis (after [5,8,12–14,16,18,20–25,27,38]).

3.4.1. *Haemogregarina rubra* n. sp. Úngari, Silva and O'Dwyer 2023

Figure 4, Table 4
(urn:lsid:zoobank.org:act:F7843DF8-7EE4-4C2D-BEA5-CC64799303CA)

Through morphological and morphometric analysis of the blood smears, this species was identified in one specimen of *Podocnemis unilifis*.

Type host. *Podocnemis unifilis* Troschel 1848 (Podocnemidae), tracajá, yellow-spotted river turtle.

Type locality. Free-living environment: Sandbanks of the Vermelho River, municipality of Britânia, Goiás state, Brazil (15°10′44.82″ S, 51°9′58.48″ W).

Site of infection. Peripheral blood erythrocytes.

Type material. Hapantotype, blood smears from *P. unifilis* were deposited at the National Instituto of Amazonian Research (INPA), Manaus, AM, Brazil **[INPA 030]**

Etymology. The specific epithet is derived from the Latin word, *rubra* (=red) in reference to the locality of where the host was captured, the Vermelho River. The name Vermelho River is related to the reddish color of the water.

Gene sequence. The 18S ribosomal RNA gene sequences were deposited in GenBank under accession numbers **[OQ377557]**.

Description. Measurements and parasitaemia are shown in Table 4. Trophozoites, gamonts with vacuoles, and two mature gamonts morphotypes were observed.

Trophozoites (Figure 4A): small, with a "comma" shape, whitish stained cytoplasm, and a rectangular nucleus occupying $\frac{1}{4}$ of the parasite and always found close to the rounded end. In some cases, cytoplasmic vacuoles are visible.

Immature gamont (Figure 4B): oval, containing curved ends, the nucleus occupying half of the parasite's body is situated to one side. Cytoplasmic vacuoles were observed in some cases.

Pre-meront (Figure 4C): body rounded on one end and slightly tapered to the other end. Nuclear chromatin is concentrated at the rounded end with a single vacuole located at the tapped end of the parasite. Parasitophorous vacuole visible, staying dark purple.

Mature gamonts (Figure 4C,D): Two morphotypes were observed, smaller and larger mature gamonts. The mature gamonts (Figure 4C) have rounded sides, with a bluish stained cytoplasm, containing small vacuoles, and are enclosed in a parasitophorous vacuole. A small, recurved tail is sometimes visible in the posterior end of the parasite. The nucleus is rectangular and centrally positioned, with a slight displacement to one end. The larger gamonts (Figure 4D) are longer and more robust, with one end larger than the other one. The parasite is enclosed in a dark and dense, purple-stained parasitophorous vacuole, with a small, recurved tail visible in the posterior end of the parasite. They possess a rectangular-shaped nucleus that extends the width of the parasite, staining dark purple, and displaced to the larger anterior end. The nucleus divides the parasite in two; on the anterior side, the cytoplasm stains light purple and on the posterior side, the cytoplasm is granulated staining dark purple. Mature gamonts cause hypertrophy to the host erythrocyte, with displacement of the host cell nucleus to one of the extremities.

Remarks. The most obvious character obtained from *Haemogregarina rubra* n. sp. are the "comma" shape trophozoite (Figure 4B) and immature gamont morphotypes

(Figure 4B): the first, small, and the second, with a nucleus occupying half of the parasite's body; and the immature with vacuoles (Figure 4C) with the nuclear chromatin dispersed throughout the parasite's body, and a parasitophorous capsule stained in dark-purple. Based on the morphology and morphometry of peripheral blood stages from *P. unifilis*, *H. rubra* n. sp. does not conform morphologically to any of the currently recognised species described from Brazil, namely *H. podocnemis*, *H. embaubali,* and *H. karaja*; or to any other species of *Haemgregarina* described from chelonids globally.

In comparison between *H. rubra* n. sp. and *H. podocnemis*, some important differences are evident: trophozoite from *H. podocnemis* always has one vacuole at the tapered end of the parasite, and nuclei are non-evident or dispersed throughout the parasite. In addition, the measurements are bigger than *H. rubra* n. sp.: 8.78 µm long × 4.06 µm wide and 23.19 μm^2 in area. Another important difference is the immature gamonts with vacuoles, which have no similar morphology in *H. podocnemis*.

Regarding *H. embaubali*, in the original description, no trophozoite and no vacuolated gamonts were reported. In addition, the mature gamont of *H. embaubali* have a parasite's capsule well-evidenced with a great thickness, which is not-evidenced in *H. rubra* n. sp.

In regards to *H. karaja*, only immature gamonts and meronts were reported, differently from *H. rubra* n. sp. where no meronts were reported for morphological and morphometric comparison between the two species.

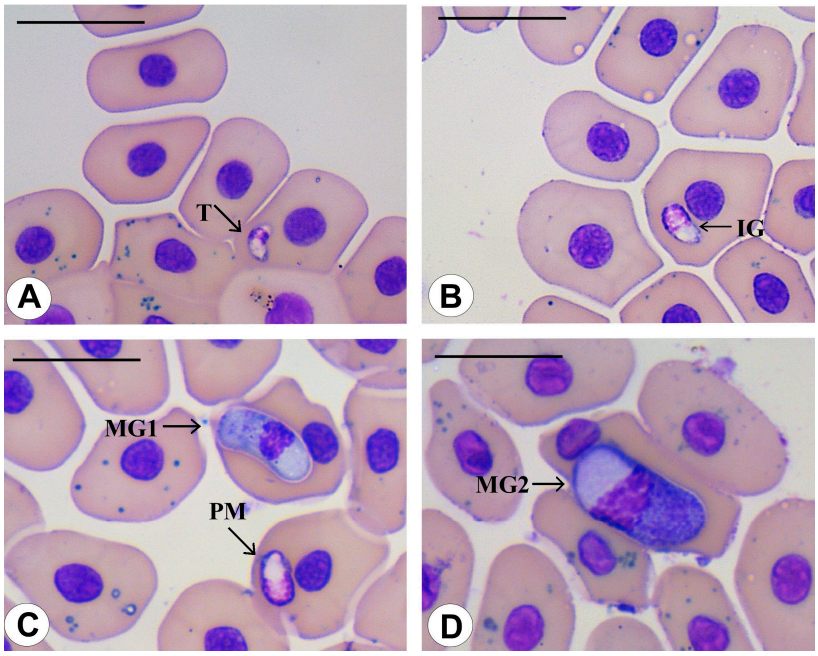

Figure 4. (**A**–**D**): Presence of developmental stages of *Haemogregarina rubra* n. sp. in the blood smears of *P. unifilis* from Goiás State, 2017. Scale: 20 µm. T: trophozoite; IG: immature gamont; PM: pre-meront with vacuole; MG1: mature gamont 1; MG2: mature gamont 2.

3.4.2. *Haemogregarina araguaiensis* n. sp. Úngari, Silva and O'Dwyer 2023

Figure 5, Table 4

(urn:lsid:zoobank.org:act:F7843DF8-7EE4-4C2D-BEA5-CC64799303CA)

Through morphological and morphometric analysis of the blood, this species was identified in two *Podocnemis unifilis.*

Type host. *Podocnemis unifilis* Troschel 1848 (Podocnemidae), tracajá, yellow-spotted river turtle.

Type locality. Free-living environment: Sandbanks of the Vermelho River, municipality of Britânia, Goiás state, Brazil (15°10′44.82″ S, 51°9′58.48″ W)

Site of infection. Peripheral blood erythrocytes.

Type material. Hapantotype, blood smears from *P. unifilis* were deposited at the National Instituto of Amazonian Research (INPA), Manaus, AM, Brazil **[INPA 027]**

Etymology. The specific epithet araguaiensis refers to the river (Araguaia), an important river in the Goiás State. The Red River, where the hosts were collected, flows into the Rio Araguaia.

Gene sequence. The 18S ribosomal RNA gene sequences were deposited in GenBank under accession numbers **[OQ388265, OQ377114]**.

Description. Measurements and parasitaemia are shown in Table 4. Trophozoites, pre-meronts, immature gamonts, and two mature gamont development stages were observed.

Trophozoites (Figure 5A,B): small and bean-shaped, with both ends rounded; contain loosely arranged nuclear chromatin occupying half of the parasite's body and staining purple.

Pre-meronts (Figure 5B,C,G): slightly concave with lysing of the parasite's nuclear chromatin, mainly in the extremities.

Immature gamont (Figure 5D): parasite slightly concave with bulged ends and enclosed in a parasitophorous vacuole. The parasites stained the cytoplasm whitish-purple, with vacuoles present in some cases. The nuclear chromatin is rectangular and located to one side of the parasite, staining purple.

Mature gamonts (Figure 5E–H): the smaller gamonts (Figure 5E,F) are slightly concave with rounded ends and enclosed in a parasitophorous vacuole, with the cytoplasm staining light blue. The nucleus is condensed, staining light purple, and located closer to the posterior end. A faint recurved tail is also visible in the posterior end of the parasite. The larger gamonts (Figure 5G,H), are slightly bent to one side and enclosed in a parasitophorous vacuole. A short, recurved tail is visible at the posterior end of the parasite. The parasite's nucleus, situated closer to the anterior end, is ovoid in shape and extends the width of the body, dividing the cytoplasm in two distinct halves. The larger posterior end is granulated and stains bluish-purple, while the smaller anterior end is smooth, staining purple. In addition, gamonts cause hypertrophy, with displacement of the host cell nucleus, and in some cases, cause the host cell to rupture.

Remarks. The most obvious characteristics observed were the small and ovoid-shaped pre-meronts (Figure 5B,C,G), with nuclear chromatin division observed in both ends of the parasite's body. Based on the morphology and morphometrics of peripheral blood stages in *P. unifilis, H. araguaiensis* n. sp. does not conform morphologically to *H. podocnemis*, *H. embaubali,* and *H. karaja*; or to any other species of *Haemogregarina* described from chelonids globally.

In the comparison to *H. podocnemis*, some important differences are evident. Although *H. podocnemis* also have comma-shaped trophozoites with cytoplasmic vacuoles, the condensed nuclei staining dark purple are not evidenced in *H. podocnemis*, and the cytoplasmic vacuoles are present in different places, depending on the type of trophozoite from *H. araguaiensis* n. sp. In addition, the pre-meronts from *H. podocnemis* are rounded with nuclear chromatin dispersed throughout the cytoplasm and staining purplish, differing from *H. araguaiensis* n. sp.

Regarding *H. embaubali* and *H. karaja*, all the developmental stages differ from *H. araguaiensis* n. sp. (10.53 µm long × 3.4 µm wide, with an area of 28.76 µm^2). The immature gamonts are considerably smaller, measuring 8.4 µm long × 4.8 µm wide, with an area of 36.4 µm^2 in *H. embaubali*; and 8.3 µm long × 4.4 µm wide, with an area of 34.2 µm^2 in *H. karaja*. In addition, the mature gamonts of *H. embaubali* are smaller than *H. araguaiensis* n. sp. (20.2 µm long × 9.9 µm wide, 131.9 µm^2 in area).

Regarding the comparison of *H. araguaiensis* n. sp. and *H. rubra* n. sp. (also from the present study), the trophozoites of *H. araguaiensis* n. sp. are larger, measuring 10.53 µm long × 3.40 µm wide, with an area of 28.66 µm^2, as compared to *H. rubra* n. sp., measuring 7.26 µm long × 3.50 µm wide, with an area of 19.38 µm^2.

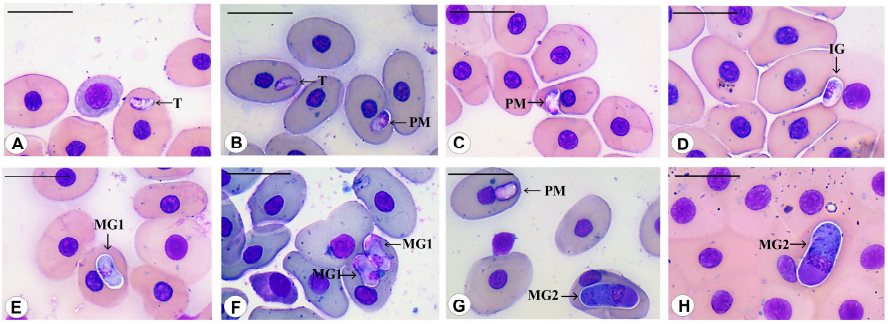

Figure 5. (**A**–**H**): Presence of developmental stages of *Haemogregarina araguaiensis* n. sp. in the blood smears of two *P. unifilis* from Goiás State, 2017. Scale: 20 µm. T: trophozoite; PM: pre-meront; IG: immature gamont; MG1: small mature gamont. MG2: large mature gamont with a recurved tail.

3.4.3. *Haemogregarina goianensis* n. sp. Úngari, Silva and O'Dwyer 2023

Figure 6, Table 4

(urn:lsid:zoobank.org:act:6232375E-27B5-4A48-A0E8-598C4F8B8321)

Through morphological and morphometric analysis of the blood, this species was identified in two fresh-water turtles: one *Podocnemis expansa* and one *Podocnemis unifilis.*

Type host. *Podocnemis expansa* Schweigger 1812 (Podocnemidae), tartaruga-da-Amazônia, giant Amazon River turtle.

Other hosts. *Podocnemis unifilis* Troschel 1848 (Podocnemidae), tracajá, yellow-spotted river turtle.

Type locality. Free-living environment: Sandbanks of the Vermelho River, municipality of Britânia, Goiás state, Brazil (15°10′44.82″ S, 51°9′58.48″ W)

Site of infection. Peripheral blood erythrocytes.

Type material. Hapantotype, blood smears from *P. expansa* and *P. unifilis* were deposited at the National Instituto of Amazonian Research (INPA), Manaus, AM, Brazil **[INPA 029]**

Etymology. The specific epithet *goianensis* refers to the State (Goiás) where the hosts were captured.

Gene sequence. The 18S ribosomal RNA gene sequences were deposited in GenBank under accession numbers **[OQ377546, OQ377555]**.

Description. Measurements and parasitaemia are shown in Table 4. Trophozoites, pre-meronts, immature gamonts, and immature and mature gamont development stages were observed.

Trophozoites (Figure 6A,B): elongated and curved, with one end slightly more rounded than the other; at the tapered end of the parasite, a cytoplasmic vacuole is always visible. The nucleus is located in the rounded end occupying the bottom half of the parasite, staining purple.

Pre-meronts (Figure 6C,F): ovoid and robust, the cytoplasm is often granulated and vacuolated, staining light purple. These stages possess a large, rounded nucleus that occupies more than half of the parasite, staining purple.

Immature gamont (Figure 6D): rounded at both ends; on some occasions, a possible cytoplasmic capsule is observed. The nucleus is rectangular and located close to one side of the parasite; with granulated chromatin, staining purple.

Mature gamonts (Figure 6E–H): two morphologies were observed. One (Figure 6E,G) is rounded at both ends; on some occasions, a possible cytoplasmic capsule is observed. The nucleus is large and rectangular, which occupies more than half of the parasite, with granulated chromatin, staining purple. The other morphology (Figure 6F–H), on the other hand, is much larger: it causes rupture or hypertrophy of the erythrocyte with a displacement of the nucleus; presents a granular cytoplasm, rectangular nucleus, and subtly shifted to the more bulged side of the parasite; and may have the same width as the parasite.

Remarks. The main characteristics obtained from this newly described species is the presence of a trophozoite (Figure 6A,B) with a vacuole on the tapered side and a well-developed nucleus; a rounded pre-meront (Figure 6C,F) with a great amount of nuclear chromatin, with small cytoplasmic vacuoles evidenced; and a large and rounded mature gamont (Figure 6E,G) with a great amount of nuclei occupying almost the entire parasite, with a parasitophorous vacuole stained dark purple. Based on the morphology and morphometry of peripheral blood stages from *P. expansa* and *P. unifilis*, *H. goianensis* n. sp. does not conform morphologically to the well-known species described in Brazil so far: *H. podocnemis*, *H. embaubali,* and *H. karaja*; or to any other species of *Haemogregarina* described from chelonids worldwide.

In the comparison between *H. goianensis* n. sp. and *H. podocnemis*, some important differences can be evidenced: Although *H. podocnemis* have their trophozoites comma-shaped with a cytoplasmic vacuole, similar in this study, the well-formed nuclei stained in dark purple are not evidenced in *H. podocnemis*. In addition, the pre-meronts from *H. podocnemis* are rounded and bigger than trophozoites, similar to *H. goianensis* n. sp., except for the great number of nuclei occupying almost all the parasites, described in *H. goianensis* n. sp.

Regarding *H. embaubali* and *H. karaja*, all the developmental stages morphologies differ from *H. goianensis* n. sp. The immature gamonts are considerably smaller, being *H. embaubali* 8.4 µm long × 4.8 µm wide, 36.4 µm^2 in area; and *H. karaja* 8.3 µm long × 4.4 µm wide, 34.2 µm^2 in area.

In the comparison among *H. goianensis* n. sp. and *H. rubra* n. sp., the trophozoites of *H. goianensis* n. sp. (8.49 µm long × 3.84 µm wide, 25.52 µm^2 in area) have higher measures than *H. rubra* n. sp. (7.26 µm long × 3.50 µm wide, 19.38 µm^2 in area). In regards to *H. araguaiensis* n. sp., the mature gamonts 2 from both species have the same morphology and morphometry; however, the trophozoites from *H. araguaiensis* n. sp. have bigger measures than *H. goianensis* n. sp., and all the others developmental stages have different morphologies and morphometries.

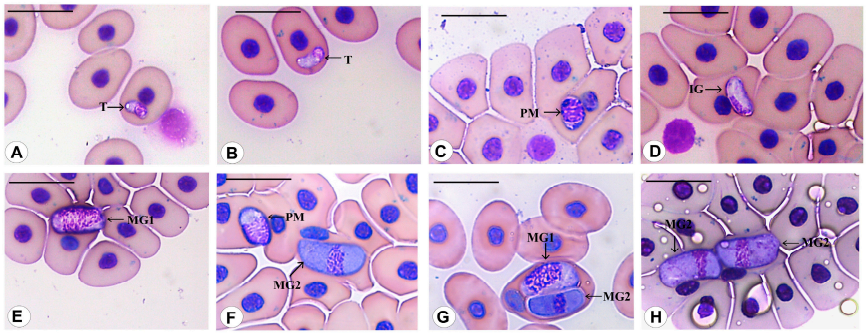

Figure 6. (**A**–**H**): Presence of developmental stages of *Haemogregarina goianensis* n. sp. in the blood smears of one *P. unifilis* and one *P. expansa* from Goiás State, 2017. Scale: 20 µm. T: trophozoite; PM: pre-meront; IG: immature gamont; MG1: mature gamont 1; MG2: mature gamont 2 with a recurved tail.

3.4.4. *Haemogregarina unifila* n. sp. Úngari, Silva and O'Dwyer 2023

Figure 7, Table 4

(urn:lsid:zoobank.org:act:0287A2A8-499A-4138-8A6F-8A57E6014B9C)

Through morphological and morphometric analysis of the blood smears, this species was identified in five specimens of *Podocnemis unilifis*.

Type host. *Podocnemis unifilis* Troschel 1848 (Podocnemidae), tracajá, yellow-spotted river turtle.

Type locality. Free-living environment: Sandbanks of the Vermelho River, municipality of Britânia, Goiás state, Brazil (15°10′44.82″ S, 51°9′58.48″ W)

Site of infection. Peripheral blood erythrocytes.

Type material. Hapantotype, blood smears from *P. unifilis* were deposited at the National Instituto of Amazonian Research (INPA), Manaus, AM, Brazil **[INPA 032]**

Etymology. The specific epithet *unifila* refers to the host's name (*P. unifilis*).

Gene sequence. The 18S ribosomal RNA gene sequences were deposited in GenBank under accession numbers **[OQ377710, OQ377566]**.

Description. Measurements and parasitaemia are shown in Table 4. Trophozoites, two morphotypes of immature gamonts, pre-meronts, meronts, and two morphotypes of mature gamonts (one smaller and one larger) were observed.

Trophozoites (Figure 7A): small and long with an oval shape, with one end more domed than the other; the nucleus occupying 3/4 of the parasite stained in purple and located close to the most domed side of the parasite.

Pre-meront (Figure 7B): rounded shape, with nuclear chromatin dispersed by the cytoplasm, and a parasitophorous capsule evidenced; the cytoplasm stained light blue is often granular and has vacuoles.

Meront (Figure 7C): a parasitophorous vacuole and nuclear division are evident, with 5–6 rounded nuclei and stained bluish-purple.

Immature gamonts (Figure 7D,G): two distinct morphologies. The first, similar to a "bean", and presenting curvature and both sides rounded, may or may not contain cytoplasmic vacuoles; a very small nucleus is located at one end of the parasite. The second morphology is small, however, a little larger than the trophozoites; they have an evident parasitophorous capsule, with a bean shape, and it is not possible to observe the nucleus.

Mature gamonts (Figure 7E,F): the smallest one is characterized by the presence of a parasitophorous vacuole and a rounded nucleus, stained in light purple and displaced to one of the ends of the parasite; both ends of the gamont are bulged, and always displace the erythrocyte nucleus due to its size. It is also possible to observe, in some cases, cytoplasmic vacuoles and a prominent recurved tail. Concerning the larger, mature gamont, a prominent recurved tail is usually observed; the parasite usually causes cellular rupture or severe deformation of the erythrocytes due to its size; its cytoplasm is blue with dark blue or purple granules, and may or may not have evident cytoplasmic vacuoles; and the nucleus is stained in light purple and dislocated to one end of the parasite. The parasitophorous capsule is often not evidenced.

Remarks. The main morphological characteristic observed from this species is the trophozoite (Figure 7A) with nuclei occupying the majority amount of the parasite's body. In addition, the morphometric values of *H. unifila* n. sp. are, so far, the smallest in comparison to the other newly described species. Based on the morphology and morphometry of peripheral blood stages from *P. unifilis*, *H. unifila* n. sp. does not conform morphologically to the well-known species described in Brazil so far: *Haemogregarina podocnemis* Úngari, Santos, O'Dwyer, da Silva, Fava, Paiva, Pinto and Cury, 2018, *H. embaubali*, and *Haemogregarina karaja*; or to any other species of *Haemgregarina* described from chelonids worldwide.

In the comparison between *H. unifila* n. sp. and *H. podocnemis*, differences can be evidenced: the trophozoite from *H. podocnemis* always has one vacuole at the tapered end of the parasite, and nuclei are not evidenced or dispersed through the parasite. In addition, the measurements are bigger than *H. unifila*: 8.78 μm long × 4.06 μm wide and 23.19 μm^2 in area. Another important difference is the mature gamont 2. The larger *H. podocnemis* mature gamont is stained pinkish with no cytoplasmic granules; the area measurements of *H. podocnemis* range from 192–300 μm^2, bigger than *H. unifila*, 150.65 μm^2.

Regarding *H. embaubali*, in the original description, no trophozoite was reported. The length measurements were bigger than *H. unifila* n. sp. from all the stages, except for meronts (10.9 μm) against 14.67 μm from this study. Moreover, some important morphological differences are evident, such as two immature gamont morphologies reported in this study against one immature gamont poorly stained whitish cytoplasm and nuclei reported from *H. embaubali*.

In regards to *H. karaja*, only immature gamonts and meronts were reported. The meronts are considerably smaller (10.9 μm long × 5.5 μm wide, 58.4 μm^2 of area) than

the meronts from this study. In addition, the *H. karaja* immature gamonts, in some cases, present the nuclei located at the middle portion of the parasite's body against the gamonts nuclei of *H. unifila*, which are non-evidenced or positioned at one extremity.

The comparison between *H. unifila* n. sp. and *H. rubra* n. sp. shows different morphologies in the trophozoite and mature gamonts 2; and the presence of gamonts with vacuoles in *H. rubra*, including the morphometric values of *H. unifila* n. sp. being smaller in all the measurements.

Moreover, the trophozoites, pre-meronts, immature gamonts, and mature gamonts morphometric values from *H. unifila* n. sp. are smaller than the morphometric values reported in *H. goianensis* n. sp. and *H. araguaiensis* n. sp.

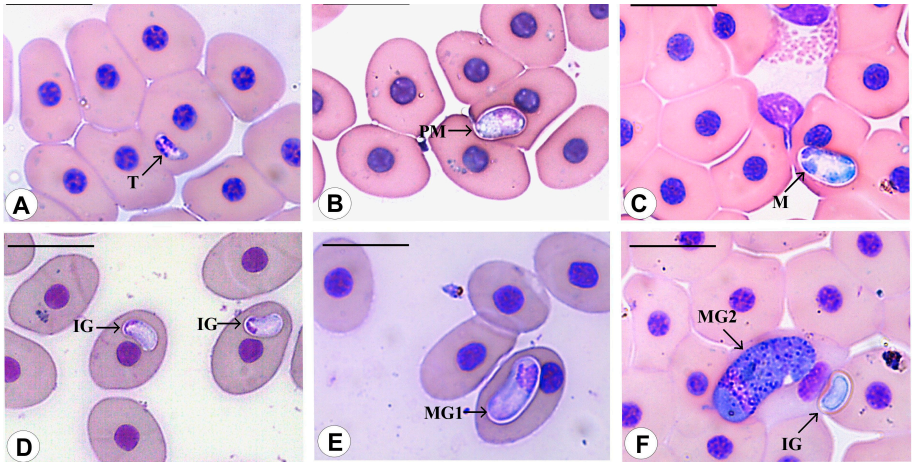

Figure 7. (**A**–**F**): Presence of developmental stages of *Haemogregarina unifila* n. sp. in the blood smears of two *P. unifilis* from Goiás State, 2017. Scale: 20 µm. T: trophozoite; PM: pre-meront; M: meront; IG: immature gamont; MG1: mature gamont 1 with a recurved tail; MG2: mature gamont 2 with a recurved tail.

3.4.5. *Haemogregarina brasiliana* n. sp. Úngari, Silva and O'Dwyer 2023

Figure 8, Table 4

(urn:lsid:zoobank.org:act:B1C01DDA-E821-4AB1-8354-BDFA706B5400)

Through the morphological and morphometric analysis of the blood, this species was identified in two *Podocnemis unifilis.*

Type host. *Podocnemis unifilis* Troschel 1848 (Podocnemidae), tracajá, yellow-spotted river turtle.

Type locality. Free-living environment: Sandbanks of the Vermelho River, municipality of Britânia, Goiás state, Brazil (15°10′44.82″ S, 51°9′58.48″ W).

Site of infection. Peripheral blood erythrocytes.

Type material. Hapantotype, blood smears from *P. unifilis* were deposited at the National Instituto of Amazonian Research (INPA), Manaus, AM, Brazil **[INPA 028]**.

Etymology. The specific epithet *brasiliana* refers to the country, Brazil.

Gene sequence. The 18S ribosomal RNA gene sequences were deposited in GenBank under accession numbers **[OQ377129, OQ377133]**.

Description. Measurements and parasitaemia are shown in Table 4. Only immature gamonts, pre-meronts, and mature gamonts were observed.

Immature gamonts (Figure 8A): small, bean-shaped, always located close to the cell nucleus; have a clean cytoplasm, dense and dark-purple stained nucleus on the convex side, and are close to one end of the parasite.

Pre-meronts (Figure 8B,C): nuclear chromatin spaced by the cytoplasm; it is possible to observe a parasitophorous capsule and they have bulgings on both sides.

Mature gamonts (Figure 8D): robust and rounded on both ends of the parasite; cytoplasm stained in light purple; nucleus stained in darker purple, located closer to one of the ends of the parasite; it is possible to observe vacuoles and cytoplasmic granules.

Remarks. The main characteristic obtained from this newly described species is the mature gamont (Figure 8A) with the concave side located close to the nuclei cell pre-meronts (Figure 8B,C) with a parasite's capsule evidenced, and only one morphology of mature gamont (Figure 8D) with cytoplasmic granules stained in dark purple. Based on the morphology and morphometry of peripheral blood stages from *P. unifilis*, *H. brasiliana* n. sp. does not conform morphologically to the well-known species described in Brazil so far: *H. podocnemis*, *H. embaubali*, and *H. karaja*; or to any other species of *Haemogregarina* described from chelonids worldwide.

The pre-meronts from *H. podocnemis* are more rounded and have the nuclear chromatin dispersed to all the cytoplasm, stained purplish, differing from *H. brasiliana* n. sp. Moreover, two morphologies of immature gamonts could be evidenced in *H. podocnemis*, against only one in *H. brasiliana* n. sp.

Regarding *H. embaubali* and *H. karaja*, the developmental stages morphologies differ from *H. brasiliana* n. sp., the mature gamonts and immature gamonts being the most different. The mature gamonts are considerably smaller than the ones from *H. brasiliana* n. sp. (21.38 µm long and 168.16 µm^2 in area), being *H. embaubali* 20.2 µm long and 131.1 µm^2 in area. In addition, a similar pattern is observed for the immature gamonts, being that *H. embaubali* measures 8.4 µm long × 4.8 µm wide, 36.7 µm^2 in area; and *H. karaja* measures 8.3 µm long × 4.4 µm wide, 34.2 µm^2 in area.

The comparison among *H. brasiliana* n. sp. and the four newly described species from this study, *H. unifila* n. sp., *H. rubra* n. sp., *H. goianensis* n. sp., and *H. araguaiensis* n. sp., show some morphological differences. The first one is regarding the mature gamonts. There is only one mature gamont observed in *H. brasiliana* n. sp., and it is similar to the morphologies of mature gamonts 2, from the other species described in this study. In addition, there is only one immature gamont morphology and non-evidenced trophozoite. In addition, *H. brasiliana* n. sp. has the biggest measures of pre-meronts in comparison to the other species.

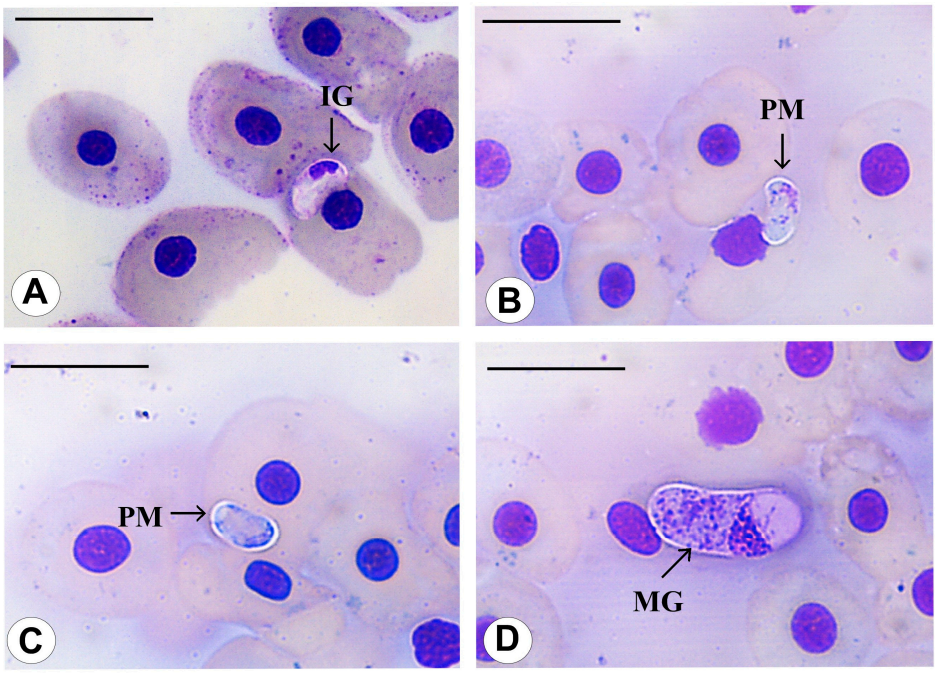

Figure 8. (**A**–**D**): Presence of developmental stages of *Haemogregarina brasiliana* n. sp. in the blood smears of two *P. unifilis* from Goiás State, 2017. Scale: 20 µm. PM: pre-meront; IG: immature gamont; MG: mature gamont.

3.4.6. *Haemogregarina tigrina* n. sp. Úngari, Silva and O'Dwyer 2023

Figure 9, Table 4

(urn:lsid:zoobank.org:act:D7FA04DC-704A-49F3-A492-8233F56AFF67)

Through morphological and morphometric analysis of the blood, this species was identified in two *Podocnemis unifilis.*

Type host. *Podocnemis unifilis* Troschel 1848 (Podocnemidae), tracajá, yellow-spotted river turtle.

Type locality. Free-living environment: sandbanks of the Vermelho River, municipality of Britânia, Goiás state, Brazil (15°10′44.82″ S, 51°9′58.48″ W).

Site of infection. Peripheral blood erythrocytes.

Type material. Hapantotype, blood smears from *P. unifilis* were deposited at the National Instituto of Amazonian Research (INPA), Manaus, AM, Brazil **[INPA 031]**.

Etymology. The specific epithet *tigrina* refers to an important lake in Britania's city, called Tiger Lake. This river is so important to the Britanensis citizens that the flag of the city has the lake's name together with a tiger drawn on it.

Gene sequence. The 18S ribosomal RNA gene sequences were deposited in GenBank under accession numbers **[OQ377558, OQ377565]**.

Description. Measurements and parasitaemia are shown in Table 4. Two morphologies of trophozoites, pre-meronts, meronts, two morphologies of immature gamonts, and mature gamonts were observed.

Merozoite (Figure 9A): small and tapered, with a large nucleus occupying almost the entire parasite and light/white cytoplasm.

Trophozoites (Figure 9B): larger than a merozoite, more robust, with a round nucleus, located at the most convex end of the parasite; and a purple cytoplasm.

Pre-meronts (Figure 9E): they have cytoplasmic vacuoles always within the most concave side and close to the cell nucleus; they have both sides rounded and the nucleus always located at one of the ends.

Meronts (Figure 9F): robust, have cytoplasmic vacuoles; it is possible to see the division of nuclear chromatin; they have a parasitophorous capsule, and always cause displacement of the cell nucleus.

Immature gamonts (Figure 9C,D,G): two morphologies. The largest is longer with one side more rounded than the other and it is possible to observe a parasitophorous capsule; the nucleus is elongated and located on the convex and more tapered side of the parasite; also, the gamonts always have the concave side closer to the erythrocyte nucleus. The smaller, immature gamonts, on the other hand, have a light purple, almost pink-stained nucleus, located at one of the ends of the parasite, which is bulged.

Mature gamonts (Figure 9G,H): they are larger, with an evident parasitophorous envelope, purple coloration, a centrally located nucleus, and with the length of the parasite width dividing the cytoplasm where one side is smooth and whitish-purple, while the other is granular and may present cytoplasmic vacuoles. These mature gamonts can cause cell disruption or hypertrophy with a displacement of the nucleus.

Remarks. The main characteristic obtained from this newly described species is the presence of bean-shaped pre-meronts (Figure 9E) with the concave side close to the cell nuclei, with chromatin nuclear division observed in both extremities of the parasite's body. Based on the morphology and morphometry of peripheral blood stages from *P. unifilis*, *H. tigrina* n. sp. does not resemble the other well-known species described in Brazil, so far: *H. podocnemis*, *H. embaubali*, and *H. karaja*; or any other species of *Haemogregarina* described from chelonids worldwide.

In the comparison with *H. podocnemis,* some important differences can be evidenced. The morphology of trophozoite in *H. podocnemis* has bigger measures (8.78 µm long × 4.06 µm wide, 23,19 µm^2 of area) than the morphology in *H. tigrina* n. sp. (7.41 µm long × 3.03 µm wide, 18.86 µm^2 in area). In addition, the meronts from *H. podocnemis* (16.43 µm long × 8.74 µm wide, 48.0 µm^2 in area) are smaller than *H. tigrina* n. sp., being 21.57 µm

long × 9.88 µm wide and 174.32 µm^2 in area. Moreover, *H. tigrina* n. sp. is characterized by only one mature gamont, differently from *H. podocnemis* mature gamonts.

Regarding *H. embaubali* and *H. karaja*, all the developmental stages morphologies differ from *H. tigrina* n. sp., the mature gamonts and meronts being the most different. The mature gamonts are considerably smaller than *H. tigrina* n. sp. gamonts, the *H. embaubali* being 20.2 µm long × 9.9 µm wide and 131.1 µm^2 in area. In addition, a similar pattern is observed for meronts, i.e., *H. embaubali* and *H. karaja*, with identical measures (10.9 µm long × 5.5 µm wide, 58.8 µm^2 in area).

According to the comparison among *H. tigrina* n. sp. and the four newly described species from this study, *H. unifila* n. sp., *H. rubra* n. sp., *H. goianensis* n. sp., and *H. araguaiensis* n. sp., some morphological differences were observed. The first one is regarding the mature gamonts. There is only one mature gamont observed in *H. tigrina* n. sp. and it is similar to the morphologies of mature gamonts 2, from the other four species described. This result is similar to that observed in *H. brasiliana* n. sp., with only one morphology of mature gamont evidenced. Moreover, in comparison to the other five newly described species, the parasite's capsule is more apparent and thick, and the measures are much bigger. In addition, only *H. tigrina* n. sp. and *H. unifila* n sp. have meronts observed, the measures of intraerythrocytic meronts from *H. tigrina* n. sp. being bigger than *H. unifila* n. sp.

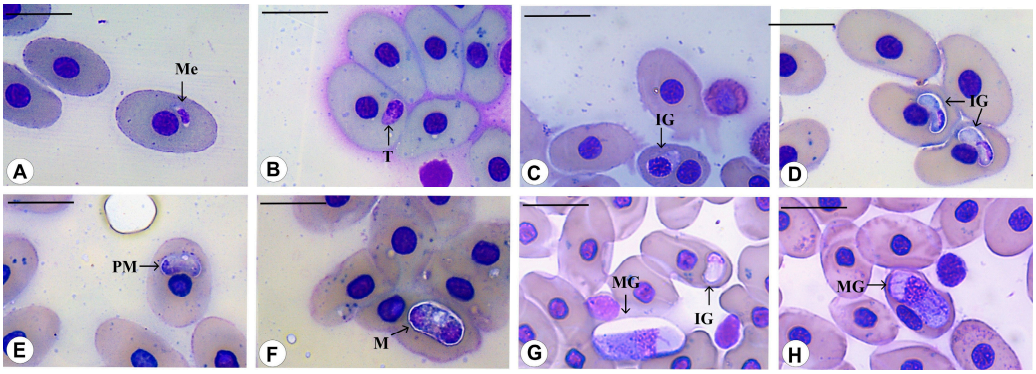

Figure 9. (**A**–**H**): Presence of developmental stages of *Haemogregarina tigrina* n. sp. in the blood smears of two *P. unifilis* from Goiás State, 2017. Scale: 20 µm. T: trophozoite; PM: pre-meront, M: meronts; Me: merozoite; IG: immature gamont; MG: mature gamont.

4. Discussion

There are eight species of turtles included in the family Podocnemididae Cope 1888: *Erymnochelys madagascariensis* (Grandidier, 1867); *Peltocephalus dumerilianus* (Schweigger, 1812); *Podocnemis erythrocephala* (Spix, 1824); *Podocnemis sextuberculata* Cornalia, 1849; *Podocnemis vogli* Müller, 1935; *Podocnemis lewyana* Duméril, 1852; *Podocnemis expansa* (Schweigger, 1812); and *Podocnemis uniflis* (Troschel, 1848) [50,51]. Among these species, *P. expansa* (the giant Amazon River turtle) is the largest freshwater turtle in South America and is widely distributed in the tributaries of the Orinoco, Essequibo, and Araguaia/Tocantins Basins, as well as the outflows of the Amazon River [50]. Regarding *P. unifilis* (yellow-spotted river turtle), this turtle is considered a medium-sized species, with the widest geographic distribution among the family. This animal can be found in rivers and lakes in the Amazon basin and Orinoco, occurring in Colombia, Venezuela, Ecuador, Peru, Bolivia, Guyana, French Guiana, Suriname, and Brazil, in this case, occurring in the states of the North region, Goiás and Mato Grosso [52].

Podocnemis expansa and *P. unifilis* are considered important representatives of the chelonian fauna in Brazil, where their meat, viscera, and eggs serve as food for local communities; and their hooves are used as adornment and household items. However,

this culture and custom of consuming these animals has decreased their numbers in their natural habitat [53,54].

According to Salera Jr. et al. [54], although there are numerous areas where these species are protected and managed by the RAN (Center for Conservation and Management of Reptiles and Amphibians), an agency linked to IBAMA (Brazilian Institute for the Environment and Renewable Natural Resources), there is a scarcity of studies focusing on these animals. The number is even lower regarding their parasites. In this study, 100% of the *P. expansa* (3/3) and *P. uniflis* (31/31) screened were positive for haemogregarine protozoans. Correa et al. [18] described two new *Haemogregarina* species infecting 50 (66%) *P. expansa* and 6 (100%) *P. sextuberculta*. In another study, conducted by Oliveira et al. [31], 7 (100%) *P. expansa* were positive for these parasites.

In the present study, the highest parasitaemia was recorded in the turtles, *P. expansa* (13.97%) and *P. unilifis* (12.49%). The second highest parasitaemia levels reported in turtles from Brazil were in *P. uniflis* at 6% [27] and *P. expansa* at 3% [14]. Both a high prevalence and parasitaemia in freshwater turtles from Brazil can be due to the quality of the environment, a stable transmission route in the region, and the persistence of haemogregarine infection for prolonged periods of time in the vertebrate host [2,55].

Notwithstanding, leeches are considered the vectors of species of *Haemogregarina*, and they are extremely susceptible to environmental changes and pollution [55]. The exposure of leeches to pesticides has negative effects on egg production, growth, feed, and survival rate [55]. Thus, haemogregarine infections may occur continuously and maintain high levels of parasitaemia in an environment suitable for leeches [14]. In addition, Siddal and Desser [24], studying the prevalence and intensity of *Haemogregarina balli* on three species of turtles from Ontario, reported that the interval of a significantly higher intensity of gamonts coincided with the major period of blood feeding by the vector, *Placobdella ornata*. Thus, the high parasitaemia found in the present study may be related to the presence of leeches.

In Brazil, Campos-Brites and Ratin [29] reported a higher prevalence (37.5%) of species of *Haemogregarina* infecting *Phrynops geoffroanus* in an urban area, where leeches were found, in comparison to an agricultural area (15.4%); these findings correlate to the water quality of the Uberabinha River.

Regarding the phylogenetic analyses, it was possible to identify *Haemogregarina* as a paraphyletic group. The new Neotropical Clade observed in this study was observed as a sister group to *Dactylosoma* Clade, while the *Haemogregarina* Fish Clade and *Haemogregarina* Clade in chelonians from European, South Africa, and North America shared a common ancestral, and constitute a sister group to Hepatozoidae and Karyolysidae isolates. Moreover, the well-supported clade with only Neotropical isolates from chelonians was also observed in the study carried out by Correa et al. [18]. In addition, the topology observed in our study also indicates that *Haemogregarina* comprised a polyphyletic group, with the presence of three well-supported clades. The first comprises Neotropical turtles; the second comprises fish hosts; and the last one comprises turtles from Europe, South Africa, and North America.

In the Neotropical Clade, six undescribed species and *H. embaubali* [18] were found in this study. This high diversity may be related to the stable habitat environment, shared by both the vertebrate host and vector, facilitating the life-cycle pathway. In addition, some species identified in this study were found infecting two different species of *Podocnemis*, which can be explained because both turtle species occupy the same niches and microhabitats, and are infested by leeches. Moreover, *H. embaubali* was described by Correa et al. [18] in *P. expansa* captured from North regions, Xingu River at Para State, and Araguaia River Basin at Tocantins State; on the other hand, we reported this species' infection in turtles from Midwest Regions, Mato Grosso, and Goiás States from Araguaia Basin. The Xingu and Tocantins–Araguaia ecoregions are part of the Western Shield of Brazil, known for sharing aquatic fauna lineages [56–59]. Thus, one hypothesis for *H. embaubali* spread is the capacity of *P. expansa* to migrate up to hundreds of kilometers in search of suitable feeding and nesting sites [58].

In addition, the second phylogenetic analysis (Figure 2), constituted by only *Haemogregarina* isolates from turtles and fish, revealed three well-supported clades. The first comprises isolates from Neotropical turtle hosts (Brazil and Colombia). This monophyletic clade was also reported by Correa et al. [18]. Another hypothesis for this new clade formation is based on a closer evolutionary relationship and greater specificity regarding the invertebrate host (and vector) than the vertebrate host. In addition, only two genera of hosts were comprised in this Clade, *Rhinoclemmys* and *Podocnemis*; in addition, both have similar reproductive and ecological characteristics, occupying the same niches and microhabitats [18,60,61]. Therefore, these conditions can facilitate hemogregarine transmission by vector sharing and host-switching events [18,62].

In the original description of *H. embaubali*, the authors only detected immature gamonts, meronts, and mature gamonts [18]. Nevertheless, in our study, in addition to the molecular identification of *H. embaubali* in 20 freshwater turtles, two *P. expansa,* and 18 *P. unifilis* (Figure 3), we also observed trophozoites, pre-meronts, meronts, gamonts with vacuoles, immature gamonts, and mature gamonts.

5. Conclusions

In conclusion, this study contributes to the diversity of Brazilian fresh-water turtles' blood parasites, with the discovery and description of six new species, and the identification of *H. embaubali* in a new host, *P. unifilis*, with tissue merogony reported. In addition, it is important to point out some hypotheses that have been raised in this study regarding the proximity of the *Hemogregarina* species in Neotropical turtles, as well as the high prevalence found in the *P. unifilis* and *P. expansa* turtles.

Author Contributions: Conceptualization, L.P.Ú., A.L.Q.S., and L.H.O.; methodology, L.P.Ú. and A.L.Q.S.; software, L.P.Ú.; validation, L.H.O., R.J.S., A.L.Q.S., and L.P.Ú.; formal analysis, L.P.Ú.; investigation, L.P.Ú.; resources, L.H.O. and R.J.S.; data curation, L.P.Ú.; writing—original draft preparation, L.P.Ú.; writing—review and editing, A.L.Q.S., R.J.S., and L.H.O.; visualization, L.P.Ú.; supervision, L.H.O.; project administration, L.H.O.; funding acquisition, L.H.O. and R.J.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Ethics Committee on Animal Use of the Institute of Biosciences by Universidade Estadual Paulista "Júlio de Mesquita Filho" (CEUA–UNESP, protocol number 1061), and the Authorization and Information System in Biodiversity (SISBIO, process no. 61940).

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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