Parasitology Research (2018) 117:1535–1548
https://doi.org/10.1007/s00436-018-5817-7

ORIGINAL PAPER

Haemogregarina podocnemis sp. nov.: description of a new species of *Haemogregarina* Danilewsky 1885 (Adeleina: Haemogregarinaidae) in free-living and captive yellow-spotted river turtles *Podocnemis unifilis* (Testudines: Podocnemididae) from Brazil

Letícia Pereira Úngari[1,2] · André Luiz Quagliatto Santos[3] · Lucia Helena O'Dwyer[2] · Maria Regina Lucas da Silva[2] · Natália Nasser de Melo Fava[1] · Guilherme Carrara Moreira Paiva[3] · Rogério de Melo Costa Pinto[4] · Márcia Cristina Cury[1]

Received: 31 July 2017 / Accepted: 16 February 2018 / Published online: 22 March 2018

Abstract

Based on morphological, morphometric, and molecular data, we describe a new hemoparasite of the genus *Haemogregarina* Danilewsky 1885, isolated from the Brazilian aquatic turtle *Podocnemis unifilis* (Testudines: Podocnemididae). The new species, *Haemogregarina podocnemis* sp. nov. (Apicomplexa: Haemogregarinidae), is characterized by small trophozoites with a single cytoplasmic vacuole on one side; pre-meronts with nuclear chromatin dispersed in the cytoplasm, with or without cytoplasmic vacuoles; meronts that are usually broad and slightly curved (kidney-shaped), with an average of eight small rectangular nuclei; immature gamonts (bean-shaped) with two morphological types: one with nuclear chromatin dispersed in the cytoplasm and the other with nuclei in the middle of the cell; mature gamonts of two morphological types: one with a length equal to or greater than that of the erythrocyte and the width of the nuclei similar to that of the hemoparasite and the other smaller than the erythrocyte with the width of the nuclei less than that of the hemoparasite. This is the first hemogregarine species described that infects the Brazilian turtle *Po. unifilis*. These findings highlight the need for further studies of *Haemogregarina* spp. to better determine the biodiversity of this understudied parasite group.

Keywords Haemogregarina · *Podocnemis unifilis* · Turtle

Introduction

Thirty-six turtle species of Brazilian reptiles are recognized (Bérnils and Costa 2012). There are only five reports of hemoparasites in these animals: *Haemoproteus peltocephalus* and *Haemoproteus geochelonis* in *Peltocephalus dumerilianus* and *Chelonoidis denticulata* (Lainson and Naiff 1998), *Haemogregarina* sp. in *Phrynops geoffroanus* (Campos-Brites and Rantin 2004), *Haemogregarina* sp. in *Podocnemis unifilis* (Soares et al. 2014), *Haemogregarina* sp. in *Podocnemis expansa* (Picelli et al. 2015), and *Haemogregarina* sp. in *Ph. geoffroanus* and *Po. expansa* held in captivity (Pessoa et al. 2016).

Hemogregarines form a group of about 400 species of adeleid blood parasites. In a review of the group, Levine (1988) placed 300 species in the genus *Haemogregarina*. Later, Siddall (1995) designated only 19 as *Haemogregarina* sensu stricto. Since then, species have been added and

Section Editor: Leonhard Schnittger

✉ Letícia Pereira Úngari
letspungari@hotmail.com; leticia.p.ungari@gmail.com

[1] Laboratório de Parasitologia, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Campus Umuarama, Uberlândia, Minas Gerais, Brazil

[2] Instituto de Biociências, Campus de Botucatu, Departamento de Parasitologia, UNESP-Univ Estadual Paulista, Distrito de Rubião Junior, Botucatu, São Paulo, Brazil

[3] Laboratório de Ensino e Pesquisa em Animais Silvestres, Faculdade de Medicina Veterinária, Universidade Federal de Uberlândia, Minas Gerais, Brazil

[4] Faculdade de Matemática-FAMAT, Centro de Ciências e Tecnologia, Universidade Federal de Uberlândia—UFU, Uberlândia, MG, Brazil

removed from this list. Previously, two species on the list were observed in snakes. In addition, *H. fitzsimonsi* was recently reassigned as *Hepatozoon fitzsimonsi* and *H. parvula* as *Hemolivia parvula* (Cook et al. 2014, 2015).

Haemogregarina species (Apicomplexa: Adeleina: Haemogregarinidae) have a heteroxenous lifecycle (Levine 1988), with vertebrate hosts such as fish and turtles, and vectors such as gnathiid isopods and leeches of the families Glossiphonidae, Ozobranchidae, and Pisicolidae (Siddal and Desser 1993, 2001; Siddall 1995; Davies et al. 2004).

In turtles, *H. balli* has the best-described lifecycle in the genus. This is a parasite found in *Chelydra serpentina* and has the leech *Placobdella ornata* as vector (Siddall and Desser 1991). Transmission occurs through blood hematophagy by leeches, with inoculation of sporozoites by the proboscis. Sexual development occurs in the vector, where gametogenesis, zygote formation, and monosporoblastic oocyst formation takes place. Sporozoites invade the proboscis of the leech and are inoculated into the turtle during feeding. In the vertebrate host, the sporozoites pierce the wall of the intestine and lodge in organs such as the lungs, spleen, and liver, where they form pre-erythrocytic meronts, and the gametocytes form in the last part of the cycle (Siddall and Desser 1991, 1992; Davies and Johnston 2000).

Diagnosis by optical microscopy was considered for a long time as a gold standard; visual detection on blood smears is commonly used to identify hemoparasites such as the hemogregarines (Sehgal et al. 2005). Nowadays, the molecular technique of polymerase chain reaction (PCR) has been explored and proved to be useful in the identification and characterization of genus and species of hemogregarines. Perkins and Keller (2001), Moço et al. (2002, 2012), Haklová et al. (2013), O'Dwyer et al. (2013), and Maia et al. (2014) detected *Hepatozoon* spp. in lizards and snakes by PCR. Pessoa et al. (2016) used the primer pair HEMO1/HEMO2 described by Perkins and Keller (2001) to detect *Haemogregarina* sp. in captive turtles. In addition, Maia et al. (2016) used the primers HepF300/Hep900 (Ujvari and Marques 2005) to detect hemogregarines in reptiles, and, thus, identified *Haemogregarina* spp. in lizards from Oman. Conventional PCR using primers ER/EF (Kvičerová et al. 2008) from 18S rRNA gene has been conducted in Southeast Asia and western Palearctic freshwater turtles targeting hemogregarines, such as *Haemogregarina* sp. (Dvoráková et al. 2013, 2015). All the primers described above target the 18S rRNA gene of the hemoparasites. The present study is the first to perform conventional PCR targeting 18S rRNA gene of hemogregarines in free-living Brazilian turtles.

In a natural environment, reptiles parasitized with hemogregarines are usually asymptomatic. However, if the parasitemia is high, there are consequences for the animal (Crawford 2008). The damage caused by these hemoparasites usually worsens in captivity, where the animal is under stress and becomes immunosuppressed, and the infected animal may present severe clinical conditions and possible secondary infections (Møller 1997; Olsson et al. 2005). In the literature, there are reports of hemolytic anemia, apathy, weight loss, and altered breeding and mating patterns (Møller 1997; Amo et al. 2005; Thrall et al. 2007) in response to infection. In addition, Oppliger and Clobert (1997) observed that parasitized lizards have reduced post-autotomy regeneration.

In the present study, free-living and captive turtles were screened for the presence of hemogregarines using both morphological and molecular techniques. We also explored the association between prevalence and turtle host origin (free living/captive) and physical variables of the host such as sex and age (adult/juvenile/new hatchling).

Materials and methods

Authorization

The project was submitted to and approved by the Ethics Committee for Animal Use (CEUA) at the Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, (protocol 032/16) and by the Biodiversity Information and Authorization System (SISBIO) (Protocol 51398-1).

Area and study population

Turtle species were collected from two sites (Table 1). (1) Laboratory of Research and Study of Wild Animals (LAPAS), Faculty of Veterinary Medicine, Federal University

Table 1 Number of samples collected from species of free-living and captive turtles from the states of Minas Gerais and Goiás, in July and September 2015 and July 2016, and the positivity for *Haemogregarina podocnemis* sp. nov. observed by light microscopy

	Species	No.	P (%)
Free living	*Podocnemis unifilis*	31	31 (100)
	Podocnemis expansa	1	0
Total		32	31 (96.87)
Captivity	*Podocnemis unifilis*	7	5 (71.43)
	Trachemys dorbigni	23	0
	Trachemys elegans	5	0
	Chelonoidis sp.	4	0
	Phrynops geoffroanus	5	0
	Kinostemon scorpioides	2	0
Total		46	5 (10.87)
Total of chelonians		78	36 (46.15)
Total of *Po. unifilis*		38	36 (94.73)

No. total of samples from each species analyzed, *P* positivity for *Haemogregarina podocnemis* sp. nov. observed from each species analyzed

of Uberlândia, Minas Gerais, Brazil (coordinates: 18°53′09.4″ S 48°15′29.5″W). Samples were collected from 46 turtles between July and September 2015. The individuals were considered captive, living in an indoor environment with adequate sanitary management and constant monitoring by veterinarians and biologists. They were born in or brought to LAPAS by the environmental police and/or the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA). The animals brought following seizure underwent clinical and laboratory tests, were dewormed, and if necessary were treated for disease. When possible, they were reintroduced into nature; otherwise, they remained in a captive environment. (2) The sand banks of Red River in Britânia city and the surrounding area (coordinates 14°57′08,4″S 51°06′30,7″W). Samples were collected from 32 turtles in July to September 2015 and in July 2016. These turtles were considered free-living because they naturally inhabited this region.

Containment and collection of blood samples

For the blood collection, the animals were physically restrained with appropriate equipment following adequate norms (Malvasio et al. 2002; Portelinha et al. 2013). The free-living turtles were captured by fishing with barbless fishing hooks (Portelinha et al. 2013) and tagged by carapacial perforation (Malvasio et al. 2002). The animals held in captivity were captured manually by the carapace and immobilized by the physical containment (Picelli et al. 2015).

During the sampling period, sex (male/female) was determined based on sexual dimorphism, and age (adult/juvenile/new hatchling) of the turtles was estimated as described by Araújo et al. (2013). In addition, the animals were visually inspected for ectoparasites such as leeches.

Approximately 2 mL of blood was collected by cervical paravertebral venous puncture (Zippel et al. 2001). This sample was divided into two aliquots. One was used for two to five blood smears performed at the collection site. The other volume was placed in a polystyrene tube containing EDTA as an anticoagulant and stored at − 20 °C until molecularly analyzed in the Laboratory of Parasitology of the Institute of the Biomedical Sciences Institute at the Federal University of Uberlândia.

Blood smears and morphological and morphometric analysis

Blood smears were fixed with absolute methanol and stained with 10% Giemsa (Eisen and Schall 2000). After staining, the blood smears were examined by optical microscopy (Olympus Optical Co.) at objectives of × 400 and × 1000 magnification. The morphology of haemogregarine developmental stages were analyzed based on previous descriptions (Telford 2009; Dvoráková et al. 2013, 2015; McAllister et al. 2014; Soares et al. 2014). The morphometric analysis involved measurements of the width, length, and area of the nuclei and body of the hemoparasite. Whenever possible, at least 50 parasites of each individual were measured and compared. The parasitemia was calculated as the percentage of infected erythrocytes observed in 2000 cells (Godfrey Jr et al. 1987).

Molecular analysis

PCR was used to confirm the presence of hemogregarines detected by light microscopy. DNA was extracted using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare) following the manufacturer's instructions.

To amplify 18S rRNA gene fragments, primers HepF300/Hep900 (Ujvari and Marques 2005), which are specific for apicomplexan parasites, were used. PCR was carried out in a final volume of 25 μL, including 1 μL each of 10 pmol primer, 12.5 μL of 2× Go Taq Green Master Mix (Promega), and 3 μL of extracted DNA, with nuclease-free water accounting for the remaining volume. PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min. As a positive control, blood samples of *Po. expansa* turtle previously diagnosed with *Haemogregarina* sp. and provided by Prof. Dr. Lúcio André Viana Dias were used, and for negative controls, PCR was carried out without extracted DNA, only with nuclease-free water. All reactions were performed in a Mastercycler Pro thermocycler (Eppendorf, Brazil), and resulting products were visualized as bands following 1.5% agarose gel electrophoresis and staining with Gel Red. Gels were observed using an ultraviolet transilluminator.

The products of interest were purified by adding 2 μL of ExoSAP IT enzyme (GE Healthcare) to 5 μL of each PCR product according to the manufacturer's recommendations. DNA sequences of the products were determined using a model 3500 Genetic Analyzer capillary sequencer (Applied Biosystems) and a BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3.1 (Applied Biosystems) according to the manufacturer's recommendations.

Electropherograms of the forward and reverse autogenerated sequences were used to generate a consensus sequence in Bioedit (Hall 1999). Three samples were sequenced: two from free-living turtles sampled in 2015 (MF476203) and 2016 (MF476205) and one sample from a captive turtle (MF476204). Sequences from the GenBank Database used to generate a phylogenetic tree and identify genes are listed in Table 5. The three sequences obtained in this study were compared with the others from GenBank using the MUSCLE algorithm in Geneious v.7.1.3 (Biomatters, http:www.geneious.com). For the phylogenetic analysis,

JModelTest v.2.1.10 (Dariba et al. 2012) was used to identify the evolutionary model for the maximum likelihood analysis. The model chosen based on the Akaike Information Criterion (AIC) was TPM2uf + I + G. The phylogeny of the parasites was inferred using PhyML v. 3.0 (Guindon et al. 2010) with 1000 replicate bootstraps. *Adelina grylli* (DQ096836) and *Adelina dimidiate* (DQ096835) were included as outgroup species.

Statistical analyses

The sample number was calculated based on a method described by Picelli et al. (2015), based on a 95% confidence level and a standard error of 5%. Values were considered statistically significant at a p value < 0.05. Associations between hemoparasite prevalence and turtle origin (free living/captive), prevalence and age (adult/juvenile/new hatchling), and prevalence and sex (male/female) were investigated using multiple and simultaneous comparison tests for binomial populations (Biase and Ferreira 2009). The gametocyte variables for each turtle specimen were studied by an analysis of variance (ANOVA) and Tukey's b post hoc test according to the distribution of data. The means and standard deviations were calculated for the area, length, and width of the hemoparasite nuclei and body in each morphological form in each species of turtle. Whenever possible, at least 50 parasites for each individual were measured and compared. The significance level was established as $p < 0.05$.

Results

Prevalence

The 78 turtles screened comprised seven species (Table 1). Thirty-six (46.15%) individuals, all *Po. unifilis*, were positive for hemogregarines. Of the 36 positive animals, 5/46 (10.86%) were from the captive environment and 31/32 (96.87%) were from the free environment. The association between environment type and parasite revealed a significant difference ($p = 0.0011$), with a prevalence of 31/31 (100%) in free-living and 5/7 (71%) in captive *Po. unifilis* (Table 1).

Of the 40 males screened, 16/17 (94.11%) free-living and 3/23 (13%) captive individuals were positive for hemogregarines. Of the 38 females screened, 15/15 (100%) free-living and 2/23 (8.69%) captive individuals were positive. The comparison of male and female positivity for hemogregarines was non-significant ($p = 0.2316$).

Regarding host age, of the 42 adults screened, 21/21 (100%) free-living and 5/21 (23.80%) captive individuals were positive for hemogregarines. Of the 21 juveniles analyzed, 7/8 (87.5%) free-living and none (0/13) of the captive individuals were positive. Of the 15 newly hatched individuals screened, 3/3 (100%) free-living individuals and none (0/12) of the captive individuals were positive. The comparison between host age and positivity for hemogregarines in free-living turtles were non-significant between juveniles and newly hatched ($p = 0.3068$) but significant between adult and juvenile ($p = 0.0204$) and adult and newly hatched ($p = 0.0001$). However, regarding captive turtles, it was not possible to associate host age with positivity, since all positive turtles were adults (Table 2).

The mean parasitemia intensities in infected turtles were 2.96% (1.02–4.9%), with the free-living animals showing higher intensity (4.03% [3.16–4.9%]) than the captive animals (1.53% [1.02–2.65%]).

No ectoparasites such as leeches were observed in the free-living or captive individuals.

Morphological and morphometric analyses

The developmental stages of haemogregarine trophozoites, pre-meronts, meronts, immature gamonts, and mature gamonts found in *Po. unifilis* were determined. The numbers of *Po. unifilis* at each developmental stage observed in the blood extensions of animals were the following: 15 trophozoites, 9 pre-meronts, and 5 meronts and for immature and mature gamonts, at least 50 parasites were observed per specimen. Between one and three immature gamonts were observed parasitizing a single erythrocyte (Fig. 1h). Two immature gamonts (Micro1 and Micro2) and two mature gamonts (Macro1 and Macro2) morphotypes were detected (Fig. 1). Morphometric data were obtained for the parasites at all developmental stages. However, for some, it was not possible to present the measure of the parasite nuclei because of the absence of defined nuclear chromatin (Tables 3 and 4).

Molecular analysis

PCR was performed on 35 of the 36 samples deemed positive by optical microscopy, because for one specimen, the sample of blood was of insufficient volume. From this total of PCR positive samples (35/35), three were sequenced: two from free-living turtles sampled in 2015 (MF476203—554 bp) and 2016 (MF476205—583 bp) and one sample from a captive turtle (MF476204—554 bp). The gene identity analysis of isolates from the present study, based on *Haemogregarina* spp. sequences available on GenBank, showed intragenotypic differences. The *H. sacaliae* reference sequence (KM887507) had 31 nucleotides that were distinct from those in MF476205 and 29 from those in MF476204/MF476203 (Tables 4, 5, and 6).

Table 2 Number of samples collected from free-living and captive turtles the states of Minas Gerais and Goiás, in July and September 2015 and July 2016 and the relationship between hemogregarine positivity and turtle age and sex

Chelonians			Age			Sex	
			Adult (%)	Juvenile (%)	Newly hatched (%)	Male (%)	Female (%)
Free life	Total	32	21	8	3	17	15
	Positivity	31	21 (100)	7 (87.5)	3 (100)	16 (94.11)	15 (100)
Captive	Total	46	21	13	12	23	23
	Positivity	5	5 (23.80)	–	–	3 (13.04)	2 (8.69)

In the phylogenetic tree, isolates from this study form a sister clade II with respect to the clade III comprising *H. balli* (common snapping turtle), *H. pellegrini* (big-headed-turtle), *H. sacaliae* (four-eyed-turtle), *H. stepanowi* (Caspian turtle, Spanish pond turtle, European pond turtle), and *Haemogregarina* sp. (KF257923/KF257924/KF257925) isolated from the Gabon mud turtle. This may suggest an ancient origin of *H. podocnemis* sp. nov. with respect to *Haemogregarina* spp. comprising clade III and, if parasite host switching has not taken place and a parasite host co-evolutionary relationship is assumed, might also suggest that the host turtle *Po. unifilis* represents a more ancient sister taxon to the *Haemogregarina* spp. host turtles of clade III. Two additional clades are comprised on one hand by the somewhat more distantly related *Haemogregarina* sp. (KX507247/KX507249/KX507248) isolated from the alligator snapping turtle (clade I) and on the other hand by the distantly related *Haemogregarina* sp. (KX453647/KX453590/KX453600) that have been isolated from the house geckos (clade IV) (Fig. 2).

Taxonomic summary

Phylum Apicomplexa Levine 1970

Family Haemogregarinidae (Neveu-Lemaire) Léger 1911

Genus *Haemogregarina* Danilewsky 1885

***Haemogregarina podocnemis* sp. nov.**

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

Type locality Free-living environment: Red River (coordinates 14°57′08.4″S and 51°06′30.7″W), Britânia city and surroundings, Goiás, Brazil. Captive environment: The Laboratory of Research and Study in Wild Animals (LAPAS), Faculty of Veterinary Medicine, Federal University of Uberlândia (coordinates: 18°53′09.4″S and 48°15′29.5″W), Uberlândia, Minas Gerais, Brazil.

Other localities Tapajós (4°43′14.47″S and 56°26′7.22″W) and Jamanxim (5°02′ 44.32″S and 56°26′35.73″W) rivers, located in Itaituba city, Pará, Brazil (Soares et al. 2014).

Type material Hapantotype, four blood smears from *Podocnemis unifilis* are deposited at the National 260 Institute of Amazonian Research (INPA), Manaus, AM, Brazil (nº INPA 12a, INPA 12B, INPA 12c, 261 INPA 12d).

Site of infection Blood erythrocytes.

Prevalence Of the 38 *Po. unifilis* analyzed, 36 (94.73%) individuals were positive for hemogregarines, with 31/31 (100%) from a free environment and 5/7 (71.42%) from a captive environment.

Parasitemia The parasite loads were 1.02–4.9%, with free-living animals showing higher parasitemia levels (3.16–4.9%) than the captive animals (1.02–2.65%).

Etymology The name refers to the host genus *Podocnemis.*

Vector Unknown.

Gene sequence The 18S ribosomal RNA gene sequences were deposited in GenBank under accession numbers MF476203, MF476204, and MF476205.

Hemoparasite description

***Trophozoites* (Fig. 1a–c)** Presence of a small single cytoplasmic vacuole in one side. Nuclei are not apparent. Parasite measures 8.78 ± 2.5 μm in length, 4.06 ± 0.66 μm in width, and 23.19 ± 2.09 μm^2 in area.

Pre-meronts (Fig. 1d, e) Larger than trophozoites, with nuclear chromatin dispersed throughout the cytoplasm, with or without cytoplasmic vacuoles. The parasite measures 11.3 ± 1.94 μm in length, 7.93 ± 1.65 μm in width, and 30.04 ± 1.32 μm^2 in area.

Meronts (Fig. 1f, g) Usually broad, slightly curved (kidney-shaped), with parasitophorous vacuoles and an average of eight small rectangular nuclei. Dimensions are 16.43 ± 1.83 μm in length, 8.74 ± 0.84 μm in width, and 48.0 ± 1.67 μm^2 in area.

Immature gamonts (Fig. 1h, i) Presence of parasitophorous vacuoles, curved (bean-shaped), with two morphotypes

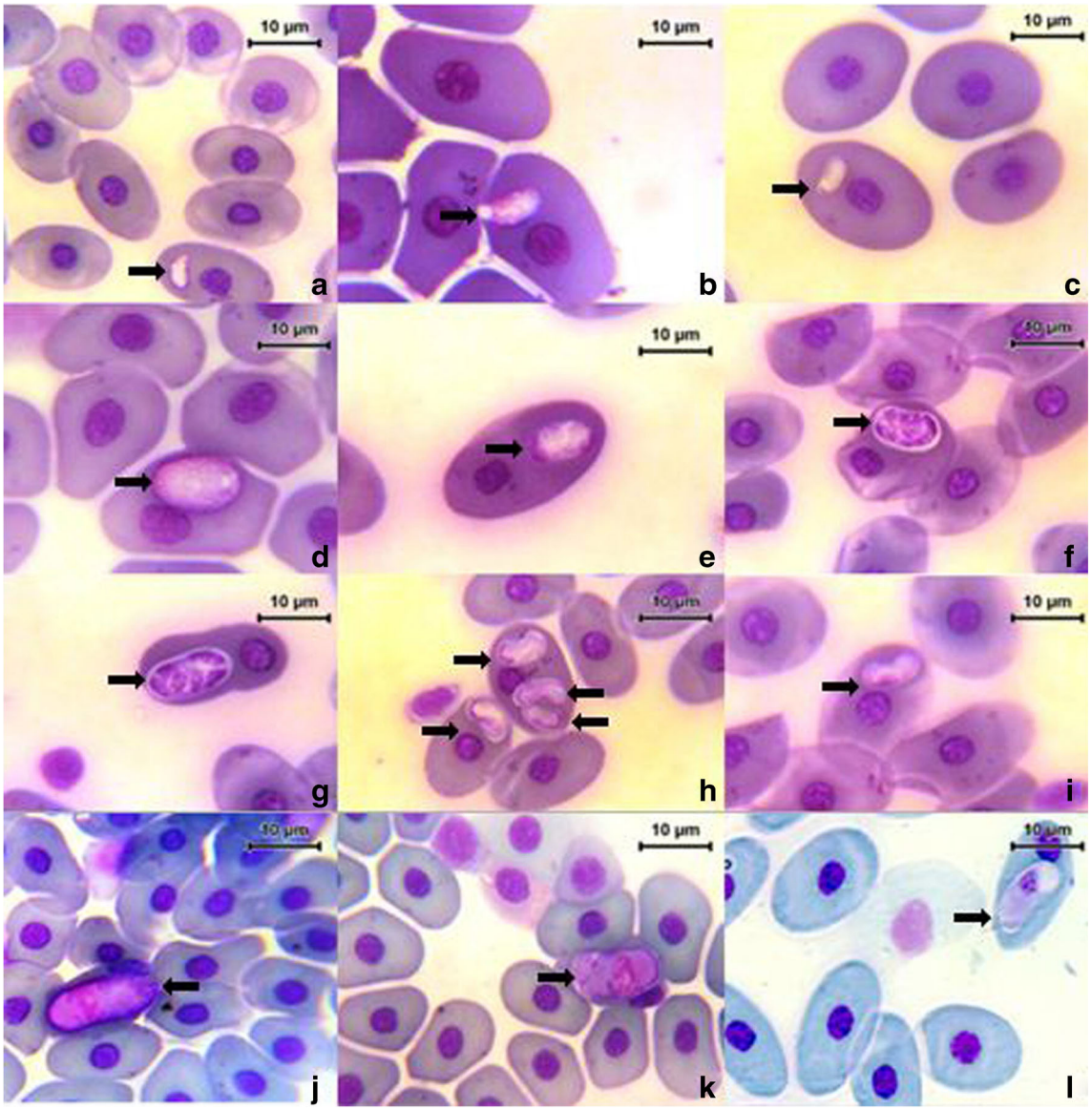

Fig. 1 Developmental stages of *Haemogregarina podocnemis* sp. nov. in the blood of *Podocnemis unifilis* (**a**–**l**). **a**–**c** trophozoite. **d**, **e** Pre-meront. **f**, **g** Meronts. **h** Immature gamonts (Micro1). **i** Immature gamonts (Micro2). **j**, **k** Mature gamonts (Macro1). **l** Mature gamonts (Macro2). Hemoparasites are indicated by arrows. Scale 10 µm

observed (Micro1 and Micro2). Micro1: Nuclear chromatin dispersed in the cytoplasm with parasites measuring 8.66 ± 1.58 µm in length, 4.3 ± 1.5 µm in width, and 15.1 ± 2.4 µm^2 in area. Micro2: Nuclei in the middle portion of the cell, with parasites measuring 11.4 ± 3.29 µm in length, 4.1 ± 0.6 µm in width, and 23.3 ± 2.4 µm^2 in area and nuclei measuring 4.96

Table 3 Morphometric data for the developmental stages of *Haemogregarina podocnemis* sp. nov. in free-living and captive turtles

	Trophozoite			Pre-meront			Meront		
	L.P	W.P	A.P	L.P	W.P	A.P	L.P	W.P	A.P
M	8.78	4.06	23.19	11.03	7.93	30.04	16.43	8.74	48.0
SD	2.5	0.66	2.097	1.94	1.65	1.32	1.83	0.84	1.67
MIN	7.15	3.34	21.08	9.66	6.37	28.50	15.06	8.2	47.12
MAX	12.68	4.65	24.33	12.41	8.44	31.46	18.89	10.42	49.89

LP parasite body length, *WP* parasite body width, *AP* parasite area, *M* mean, *SD* standard deviation, *MIN* minimum, *MAX* maximum. Linear measurements in µm and measurements of the areas in µm^2

Table 4 Morphometric data for the developmental stages of *Haemogregarina podocnemis* sp. nov. in free-living and captive turtles

	Mature gamont (Macro1)					Mature gamont (Macro2)					Immature gamont (Micro1)					Immature gamont (Micro2)				
	L.P	W.P	A.P	L.N	W.N	A.N	L.P	W.P	A.P	L.N	W.N	A.N	L.P	W.P	A.P	L.N	W.N	A.N	L.P	W.P	A.P	L.N	W.N	A.N
M	20.5	8.12	44.6	7.94	4.3	24.6	18.8	5.6	39.5	4.0	3.6	14.1	8.66	4.3	15.1	–	–	–	11.4	4.1	23.3	4.96	0.43	2.4
SD	0.82	0.76	6.01	0.76	0.0	0.9	1.3	0.8	2.9	0.6	0.3	1.74	1.58	1.5	2.4	–	–	–	3.29	0.6	2.4	1.9	0.46	1.39
MIN	19.8	6.79	30.9	6.48	3.18	19.2	17.4	4.3	37.5	3.1	2.99	11.5	4.71	2.5	9.4	–	–	–	9.45	3.5	10.7	2.13	0.03	0.85
MÁX	20.9	10.0	58.3	9.31	5.29	30.0	19.2	6.9	40.9	4.86	3.99	15.8	13.8	11	20.8	–	–	–	14.1	5.2	27.5	7.94	0.69	3.55

Linear measurements in μm and measurements of the areas in μm^2

LP parasite body length, *WP* parasite body width, *AP* parasite area, *LN* (parasite nuclei length, *WN* parasite nuclei width, *AN* parasite nuclei area, *M* mean, *SD* standard deviation, *MIN* minimum, *MAX* maximum

± 1.9 μm in length, 0.43 ± 0.46 μm in width, and 2.4 ± 1.39 μm^2 in area.

Mature gamonts (Fig. 1j–l) Deformations in erythrocytes and displacement of cell nuclei to the side. Presence of parasitophorous vacuoles, with two morphotypes observed (Macro1 and Macro2). Macro1: Length of parasite equal to or greater than the erythrocyte, with nucleus width equal to that of the hemoparasite. Parasite measures 20.5 ± 0.82 μm in length, 8.12 ± 0.76 μm in width, and 44.6 ± 6.01 μm^2 in area. Nuclei measure 7.94 ± 0.76 μm in length, 4.3 ± 0.0 μm in width, and 24.6 ± 0.9 μm^2 in area. Macro2: Parasite length is less than that of the erythrocyte. Nucleus width is less than that of the hemoparasite. Parasite measures 18.8 ± 1.3 μm in length, 5.6 ± 0.8 μm in width, and 39.5 ± 2.9 μm^2 in area. Nuclei measure 4.0 ± 0.6 μm in length, 3.6 ± 0.3 μm in width, and 14.1 ± 1.74 μm^2 in area.

Remarks The trophozoites described here had a single cytoplasmic vacuole at one end. This differs from the description by Dvoráková et al. (2013) of several vacuoles in a single trophozoite. Complementing this, Telford et al. (2009) reported the presence of nuclei, whereas in the present study, all trophozoites observed had no defined nuclei. However, Dvoráková et al. (2013) reported the absence of nuclei in *H. stepanowi*, which is consistent with that found in the present study. The morphometric means of length and width described here agree with those of other *Haemogregarina* species described in the literature. Dvoráková et al. (2013) reported these developmental stages of *H. stepanowi* in newly captured turtles in Morocco, with a mean parasite length and width of 9.6 ± 1.3 × 5.3 ± 0.5 μm. Dvoráková et al. (2015) also described *H. sacaliae* turtles, reporting a parasite size of 7.5 ± 0.9 × 2.7 ± 0.5 μm.

The pre-meronts identified in this study differ in their morphology from that cited in the literature. They are similar in length but differ in width from the description by Dvoráková et al. (2013).

Soares et al. (2014) analyzed the morphology and morphometry of primary erythrocytic meronts in *Po. unifilis* (the same turtle analyzed in this study) and reported 5–10 nuclei, with average length of 28.34 ± 2.28 μm, width of 14.505 ± 1.024 μm, and area of 73,193 ± 6.034 μm^2. The meronts in the present study were smaller and possessed 4–7 nuclei.

The immature gamonts and mature gamonts analyzed in this study were similar to those described by Soares et al. (2014), who reported two morphological characteristics of immature gamonts, named by the authors as microgametocytes, and two of mature gamonts (macrogametocytes). It should be noted that in the present study, no nuclei of one of the morphological forms of immature gamonts (Micro1) were measured because of their indefinite nuclei.

Because of the morphological and morphometric similarities between hemoparasites observed in the present study and those described by Soares et al. (2014), we could infer that

Table 5 List of sequences from GenBank database used in this manuscript to generate the phylogenetic tree and the gene identity analysis

	Isolate	Host	Locality	No. GenBank	Reference
1	*Haemogregarina podocnemis* sp. nov.	*Podocnemis unifilis*	Brazil	MF476203	This study
2	*Haemogregarina podocnemis* sp. nov.	*Podocnemis unifilis*	Brazil	MF476204	This study
3	*Haemogregarina podocnemis* sp. nov.	*Podocnemis unifilis*	Brazil	MF476205	This study
4	*Adelina dimidiata*	*Varanus albiguiaris*	Czech Republic	DQ096835	Kopečná et al. (2006)
5	*Adelina grylli*	*Gryllus bimaculatus*	Czech Republic	DQ096836	Kopečná et al. (2006)
6	*Haemogregarina* sp.	*Asaccus platyrhynchus*	Oman	KX453595	Maia et al. (2016)
7	*Haemogregarina* sp.	*Hemidactylus hajarensis*	Oman	KX453647	Maia et al. (2016)
8	*Haemogregarina* sp.	*Hemidactylus hajarensis*	Oman	KX453590	Maia et al. (2016)
9	*Haemogregarina* sp.	*Hemidactylus hajarensis*	Oman	KX453600	Maia et al. (2016)
10	*Haemogregarina* sp.	*Macrochelys temminckii*	United States	KX507248	Alhaboubi et al. (2017)
11	*Haemogregarina* sp.	*Macrochelys temminckii*	United States	KX507249	Alhaboubi et al. (2017)
12	*Haemogregarina* sp.	*Macrochelys temminckii*	United States	KX507247/KX507250/ KX507246	Alhaboubi et al. (2017)
13	*Haemogregarina* sp.	*Pelusios williamsi*	Kenya	KF257923	Dvořáková et al. (2013)
14	*Haemogregarina* sp.	*Pelusios marani*	Gabon	KF257924	Dvořáková et al. (2013)
15	*Haemogregarina* sp.	*Pelusios subniger*	Mozambique	KF257925	Dvořáková et al. (2013)
16	*Haemogregarina sacaliae*	*Sacalia quadriocellata*	Vietnam	KM887507	Dvořáková et al. (2015)
17	*Haemogregarina balli*	*Chelydra serpentina serpentina*	Canada	HQ224959	Barta et al. (2012)
18	*Haemogregarina pellegrini*	*Platysternon megacephalum*	China	KM887509	Dvořáková et al. (2015)
19	*Haemogregarina pellegrini*	*Malayemys subtrijuga*	Vietnam	KM887508	Dvořáková et al. (2015)
20	*Haemogregarina stepanowi*	*Mauremys caspica*	Iran	KF257926	Dvořáková et al. (2013)
21	*Haemogregarina stepanowi*	*Mauremys leprosa*	Algeria	KF257929	Dvořáková et al. (2013)
22	*Haemogregarina stepanowi*	*Emys orbicularis*	Bulgaria	KF257928	Dvořáková et al. (2013)
23	*Haemogregarina stepanowi*	*Mauremys caspica*	Turkey	KF992697	Kvicerova et al. (2014)
24	*Haemogregarina stepanowi*	*Mauremys rivulata*	Syria	KF257927	Dvořáková et al. (2013)
25	*Haemogregarina stepanowi*	*Emys orbicularis*	Serbia	KT749877	Ozvegy et al. (2015)
26	*Haemogregarina stepanowi*	*Emys trinacris*	Italy	KX691417	Arizza et al. (2016)
27	*Haemogregarina stepanowi*	*Emys trinacris*	Italy	KX691418	Arizza et al. (2016)
28	*Hemolivia mauritanica*	*Testudo marginata*	Greece	KF992710	Kvicerova et al. (2014)
29	*Hemolivia parvula*	*Kinixys zombensis*	South Africa	KR069083	Cook et al. (2015)
30	*Hepatozoon fitzsimonsi*	*Kinixys zombensis*	South Africa	KR069084	Cook et al. (2015)
31	*Hepatozoon musa*	*Philodryas nattereri*	Brazil	KX880079	Borges-Nojosa et al. (2017)
32	*Hepatozoon ayorgbor*	*Pyrhon regius*	Ghana	EF157822	Sloboda et al. (2007)
33	*Hepatozoon sipedon*	*Nerodia sipedon*	Canada	JN181157	Barta et al. (2012)
34	*Hepatozoon* sp. Boiga	*Boiga irregularis*	Australia	AF297085	Jakes et al. (2001)
35	*Hepatozoon* sp.	*Clethrionomys glareolus*	Spain	AY600626	Criado-Fornelio et al. (2006)
36	*Hepatozoon* sp.	*Akodon* sp.	Brazil	KU667308	Demoner et al. (2016)
37	*Hepatozoon* sp.	*Podarcis bocagei*	Spain	JX531954	Maia et al. (2012)
38	*Hepatozoon* sp.	*Podarcis vaucheri*	Marocco	HQ734794	Maia et al. (2011)
39	*Hepatozoon catesbianae*	*Pantoea agglomerans*	United States	AF176837	Mathew et al. (2000)
40	*Hepatozoon* sp.	*Rana esculenta*	Canada	HQ224960	Barta et al. (2012)
41	*Hepatozoon felis*	*Felis catus*	Spain	AY620232	Criado-Fornelio et al. (2006)
42	*Hepatozoon felis*	*Panthera leo*	India	KX017290	Rafiqi et al. (2016) unpublished
43	*Hepatozoon americanum*	*Dusicyon thous*	Brazil	AY461377	Criado-Fornelio et al. (2006)
44	*Hepatozoon americanum*	*Canis lupus familaris*	United States	AF176836	Mathew et al. (2000)
45	*Hepatozoon canis*	Fox	Spain	AY150067	Criado-Fornelio et al. (2003)
46	*Hepatozoon canis*	*Canis lupus familiaris*	Sudan	DQ111754	Oyamada et al. (2005)

they belong to the same species of *Haemogregarina*. However, Soares et al. (2014) only provided the generic placement of the parasites they described. It should be emphasized that the morphological and morphometric measures of the various haemogregarine developmental stages in turtle have not been described previously in the literature.

Based on a comparison with the morphological and morphometric features of blood developmental stages of *Haemogregarina* spp. in the literature, we conclude that Soares et al. (2014), recorded in *Po. unifilis* the same morphotypes of *H. podocnemis* sp. nov. observed in the present study. However, the authors only identified the genus of this parasite.

Table 6 The unshaded matrix (upper) shows similar nucleotides and the shaded matrix (lower) shows the different nucleotides between the isolates of *Haemogregarina* spp. from the GenBank database and those obtained in this study

Isolate	1[a]	2[a]	3[a]	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. MF476205[a]		99.6%	99.6%	93.0%	93.4%	91.5%	90.6%	90.6%	90.4%	94.1%	93.4%	93.8%	93.7%	93.8%	93.8%	93.6%	93.4%	93.8%
2. MF476204[a]	2		100%	93.4%	93.7%	91.1%	90.2%	90.2%	90.0%	94.5%	93.8%	94.1%	94.1%	94.2%	94.2%	93.6%	93.4%	93.8%
3. MF476203[a]	2	0		93.4%	93.7%	91.1%	90.2%	90.2%	90.0%	94.5%	93.8%	94.1%	94.1%	94.2%	94.2%	93.6%	93.4%	93.8%
4. KF257923	37	35	35		98.6%	92.4%	90.9%	91.3%	91.1%	98.1%	97.9%	98.3%	98.3%	98.4%	98.4%	96.2%	94.9%	95.3%
5. KF257924	35	33	33	7		91.3%	90.2%	90.2%	90.0%	98.6%	98.5%	98.6%	98.8%	98.9%	98.9%	95.8%	94.5%	94.9%
6. KX453595	45	47	47	40	46		94.9%	95.3%	95.1%	91.3%	91.1%	91.3%	91.5%	91.5%	91.5%	92.8%	92.6%	93.0%
7. KX453647	50	52	52	48	52	27		99.6%	99.8%	90.2%	89.9%	90.2%	90.4%	90.4%	90.4%	91.7%	91.7%	92.1%
8. KX453590	50	52	52	46	52	25	2		99.8%	90.2%	89.9%	90.2%	90.4%	90.4%	90.4%	92.1%	92.1%	92.5%
9. KX453600	51	53	53	47	53	26	1	1		90.0%	89.7%	90.0%	90.2%	90.2%	90.2%	91.9%	91.9%	92.3%
10. KM887507	31	29	29	10	7	46	52	52	53		98.3%	99.0%	99.0%	99.1%	99.1%	95.8%	94.5%	94.9%
11. HQ224959	35	33	33	11	8	47	54	54	55	9		98.6%	98.8%	98.9%	98.9%	95.5%	94.2%	94.4%
12. KM887509	33	31	31	7	7	46	52	52	53	5	7		99.4%	99.5%	99.5%	96.2%	94.5%	94.9%
13. KT749877	33	31	31	9	6	45	51	51	52	5	6	3		99.9%	99.9%	95.8%	94.5%	94.9%
14. KX691417	33	31	31	9	6	45	51	51	52	5	6	3	1		99.9%	95.9%	94.6%	95.0%
15. KX691418	33	31	31	9	6	45	51	51	52	22	6	3	1	0		95.9%	94.6%	95.0%
16. KX507248	34	34	34	20	22	38	44	42	43	29	24	20	22	22	22		97.5%	97.9%
17. KX507249	35	35	35	27	29	39	44	42	43	27	31	29	29	29	29	13		99.6%
18. KX507247	33	33	33	25	27	37	42	40	41	27	29	27	27	27	27	11	2	

[a] Sequences obtained from the presented study

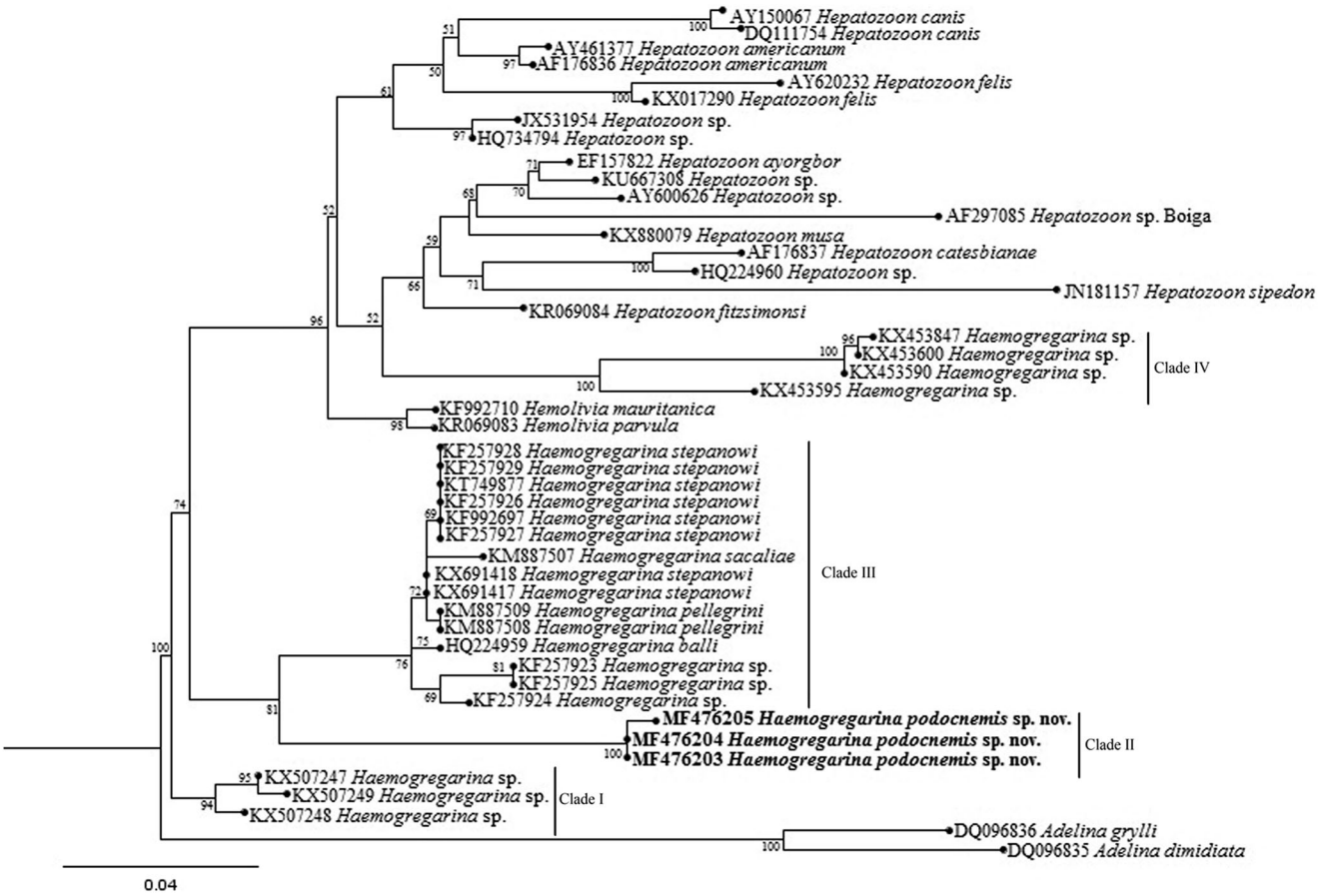

Fig. 2 Maximum likelihood (ML) phylogeny of hemogregarine species (Apicomplexa: Adeleina) based on 18S rRNA sequences. Bootstrap values are shown next to the corresponding clades (> 50%). The sequences of *H. podocnemis* sp. nov. from *Po. unifilis* in this study are highlighted. *Adelina grylli* (DQ096836) and *Adelina dimidiate* (DQ096835) form an outgroup

Discussion

Hemogregarines are the most common hemoparasites reported in reptiles (Garcia-Navarro and Pachalli 1994). In the current study, the same observation was made, as *H. podocnemis* sp. nov. was highly prevalent in the specimens screened. Data on hemoparasites in captive turtles are scarce; in Brazil, there are only two reports. Picelli et al. (2015) found that 100% of commercially bred *Po. expansa* turtles and 0% of naturally bred *Po. expansa* turtles were positive for hemogregarines. Pessoa et al. (2016) observed that 11.11% of the turtles were positive in the Zoological Foundation of Brasília, and similar results of captive animals are reported in the current study.

In the present study, the high occurrence of *H. podocnemis* sp. nov. (96.87%) in free-living turtles corroborates the observations of Jakes et al. (2001), who reported 100% *Haemogregarina* sp. prevalence in the eastern long-necked turtle (*Chelodina longicollis*) and in the Brisbane short-necked turtle (*Emydura signata*); Mihalca et al. (2002), who reported 77% prevalence in northern Australian snapping turtle (*Elseya latisternum*) and 100% prevalence in the European pond turtle (*Emys orbicularis*); and Davies and Sterrett (2011), who reported 88.9% prevalence in the common musk turtle (*Sternotherus odoratus*). Besides that, Soares et al. (2014) reported high occurrence (98%) of *Haemogregarina* sp. in free-living *Po. unifilis* turtles, similar to that observed in this study (100%).

The prevalence in captive turtles was lower than that observed in free-living turtles, presumably because free-living animals are more commonly exposed to the parasite through frequent contact with several possible vectors in the environment. Captive animals may be more easily contained and their health conditions managed. It should be noted that we collected blood from animals in the dry season, from July to September 2015 and in July 2016. This is the time females are depositing eggs on the sandbanks exposed by receding river. This results in the conglomeration of individuals in specific places, which promotes more rapid and widespread propagation of parasites in these individuals because of the feeding of haemogregarine vectors on multiple hosts (Wosniak et al. 1994; Brown et al. 2000; Coutinho 2003; Sandland and Minchella 2003).

In Brazil, *Po. unifilis* are found mainly in the Tocantins-Araguaia and Amazon Basin. *Podocnemis unifilis* (Troschel,

1848), also known as tracajá and the yellow-spotted river turtle, is broadly distributed in South America, with habitat that spans Venezuela, Colombia, Eastern Ecuador, Peru, Bolivia, French Guiana, Guyana, Suriname, and Brazil (Vogt 2008). Eggs and adult animals have been used as a food source (Ferrari 1980), and the wide distribution and abundance of the species in different basins make it difficult to estimate its conservation status. However, it is classified as vulnerable in the International Union for Conservation of Nature Red List. *Podocnemis unifilis* has the second largest body size among members of this genus in Brazil, with sexual dimorphism evident in adults. Males are smaller with larger tails and females are larger with smaller tails (Salera-Junior and Malvasio 2005). The species has a dorsoventrally flattened carapace that is brown or olive green in color and that does not exceed 70 cm in length (Ibama 1989).

Our statistical analysis did not reveal a significant association between *H. podocnemis* sp. nov. prevalence, host age (juvenile and newly hatched) ($p = 0.3068$), and host sex ($p = 0.2316$). However, McCoy et al. (2007) and Readel et al. (2008) reported higher positivity in adult female turtles. The authors suggested that this occurs because females are larger in size than males, and so there is a larger contact surface for adherence and feeding of vectors. The same difference in size can be seen from newly hatched to adult. Therefore, the significant association observed between hemoparasite prevalence from adult and newly hatched ($p = 0.0001$) and adult and juvenile ($p = 0.0204$) can also be explained by the size.

The mean parasitemia observed in free-living *Po. unifilis* (4.03%) was lower than that recorded by Soares et al. (2014), who also worked with this species of turtles. Nevertheless, our report is still the second-highest haemogregarine intensity ever registered in turtles, with the third-highest parasitemia intensity (3%) observed by Picelli et al. (2015) in *Po. expansa* turtles from the Amazon region. The present study is the second to obtain such information on *Po. unifilis* turtles. According to Soares et al. (2014), the high parasitemia and prevalence suggests stable transmission between the vertebrate host and vector (leeches). More studies must be done to better understand the lifecycle of this new parasite, as well as to identify its vector.

In captivity, under confinement stress, the reptiles are more susceptible to parasites (Telford Jr 1971). However, the haemogregarine intensity level in captive individual (1.53%) was lower than that in the wild individuals. According to Picelli et al. (2015), this pattern might be related to the different environments (conditions) and the mode of transmission of this parasite. It is possible that the captive environment provides inhospitable conditions for leech survival.

In the morphological analysis, various blood developmental stages were observed. However, immature and mature gamonts were the most prevalent. These forms are infective to vectors, and their greater presence in host blood can be explained by the collection period from July to September 2015 and in July 2016, periods that coincide with more vectors, and more blood feeding by vectors, in nature (Siddall and Desser 1991). In addition, the presence of various developmental stages, including trophozoites, pre-meronts, meronts, immature gamonts, and mature gamonts, may be related to the persistence of hemogregarines that persist in the hosts owing to the schizogonic cycle. Siroký et al. (2004) reported infection of *Hem. mauritanica* in *Testudo marginata* for a period of 1–8 years.

In Brazil, there are few reports of *Haemogregarina* spp. in turtles, and identification is based mainly on morphological observations of the blood developmental stages of the parasite in vertebrate hosts. Among the studies reported, Soares et al. (2014) performed morphological and morphometric characterization of blood developmental stages of a *Haemogregarina* sp. In addition, there have been three other studies on *Haemogregarina* spp. in Brazilian turtles, of which none provided any data on morphological and morphometric analyses. Campos-Brites and Rantin (2004) analyzed the incidence of hemogregarines in aquatic turtles of the species *Ph. geoffroanus* but did not identify the parasite to genus level. Picelli et al. (2015) analyzed the prevalence and parasitemia levels in turtles of *Po. expansa*, and Pessoa et al. (2016) classified the parasite's genus using a molecular technique, but did not sequence the PCR product.

In this study, the generic HepF300/Hep900 primer sets were used to amplify the variable region of the conservative 18S rRNA gene of the apicomplexan parasites. *Haemogregarina* spp. can be identified by sequencing the amplified PCR product, as there are no primers specific for this genus reported in the literature. Thus, the present study used these primers to confirm the generic placement as determined by the morphological analysis. Moreover, Maia et al. (2016) used the HepF300/Hep900 primers for a phylogenetic analysis of apicomplexa parasites and were able to molecularly characterize *Haemogregarina* sp. and *Hepatozoon* sp. in reptiles from Oman.

There are no sequences of *Haemogregarina* species from Brazilian turtles deposited in GenBank. The sequences available are from *H. stepanowi* (Dvoráková et al. 2013; Ozvegy et al. 2015; Arizza et al. 2016), *H. pellegrini* (Dvoráková et al. 2015), *H. balli* (Barta et al. 2012), and *H. sacaliae* (Dvoráková et al. 2015) obtained in different countries. Other sequences available in GenBank identified the genus *Haemogregarina* only. All available sequences were used in the phylogenetic analysis. Isolates of *Haemogregarina* spp. grouped in different branches, and *H. podocnemis* sp. nov. was identified as a sister group to the other isolates.

The scarcity of information for molecular assays makes species identification unlikely, particularly for Brazilian isolates. However, the morphological and morphometric characteristics observed in the present study were not identical

to those observed for *H. balli* (Barta et al. 2012), *H. stepanowi* (Dvoráková et al. 2013), *H. sacaliae* (Dvoráková et al. 2015), or *H. pellegrini* (Dvoráková et al. 2015). It is worth reminding that Soares et al. (2014) observed the same morphological and morphometric characteristics of the parasite, in the same turtle species (*Po. unifilis*) as the present study. However, the authors did not perform the molecular analysis and only identified the genus *Haemogregarina*; thus, probably, both manuscripts observed the species *H. podocnemis* sp. nov.

Based on these results, we described a new species, *H. podocnemis* sp. nov., in *Po. unifilis* from Brazil. In addition, this is the first study to use the HepF300/Hep900 PCR primers on specimens from Brazilian free-living turtles (*Po. unifilis* and *Po. expansa*) and to report the prevalence of this parasite in captive *Po. unifilis.*

Acknowledgments We thank the team at the Laboratory for Teaching and Research in Wild Animals (LAPAS) and the veterinarians at Exotic Life, Rodrigo Rabello and Matheus Rabello, for their assistance in the collection of blood from turtles. We thank Prof. Dr. Lúcio André Viana Dias and Amanda Picelli for providing a positive control for the molecular assay.

Funding information We thank Coordination for the Improvement of Higher Education Personnel (CAPES) and Research Foundation of the State of Minas Gerais (FAPEMIG) for their financial support.

Compliance with ethical standards

The project was submitted to and approved by the Ethics Committee for Animal Use (CEUA) at the Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, (protocol 032/16) and by the Biodiversity Information and Authorization System (SISBIO) (Protocol 51398-1).

References

Alhaboubi AR, Pollard DA Holman PJ (2017) Molecular and morphological characterization of a haemogregarine in the alligator snapping turtle, Macrochelys temminckii (Testudines: Chelydridae). Parasitol Res 116:207–215

Amo L, López P, Martin J (2005) Prevalence and intensity of haemogregarine blood parasites and their mite vectors in the common wall lizard, *Podarcis muralis*. Parasitol Res 96:378–381

Araújo JC, Palha MDC, Rosa PV (2013) Nutrição na Quelonicultura—Revisão. Rev Elet Nutrit 10:2828–2871

Arizza V, Sacco F, Russo D, Scardino R, Arculeo M, Vamberger M, Marrone F (2016) The good, the bad and the ugly: Emys trinacris, Placobdella costata and Haemogregarina stepanowi in Sicily (Testudines, Annelida and Apicomplexa. Folia Parasitol 63

Barta JR, Ogedengbe JD, Martin DS, Smith TG (2012) Phylogenetic Position of the Adeleorinid Coccidia (Myzozoa, Apicomplexa, Coccidia, Eucoccidiorida, Adeleorina) Inferred Using 18S rDNA Sequences. J Eukaryot Microbiol 59:171–180

Bérnils RS, Costa HC (2012) Brazilian reptiles: list of species. Soc. Bras. de Herpet. http://www.sbherpetologia.org.br. Accessed 13 March 2014

Biase NG, Ferreira DF (2009) Comparações múltiplas e testes simultâneos para parâmetros binomiais de K populações independentes. Ver Bras Biom 27:301–323

Borges-Nojosa DM, Borges-Leite MJ, Maia JP, Zanchi-Silva D, da Rocha Braga R, Harris DJ (2017)A new species of Hepatozoon Miller, 1908 (Apicomplexa: Adelerina) from the snake Philodryas nattereri Steindachner (Squamata: Dipsadidae) in northeastern Brazil Syst Parasitol 94: 65–72

Brown MJF, Loosli R, Hampel SP (2000) Condition-dependent expression of virulence in a trypanosome infecting bumblebees. Oikos 91: 421–427

Campos-Brites VL, Rantin FT (2004) The influence of agricultural and urban contamination on leech infestation of freshwater turtles, *Phrynops geoffroanus*, taken from two areas of the Uberabinha river. Environ Monit Asses 96:273–281

Cook CA, Lawton SP, Davies AJ, Smit NJ (2014) Ressignment of the land tortoise haemogregarine *Haemogregarina fitzsimonsi* Dias 1953 (Adeleorina: Haemogregarinidae) to the genus *Hepatoozon* Miller 1908 (Adeleorina: Hepatozoidae) based on parasite morphology, life cycle and phylogenetic analysis of 18S r DNA sequences fragments. Parasitology 141:1611–1620

Cook CA, Netherlands EC, Smit NJ (2015) First *Hemolivia* from southern Africa: reassigning chelonian *Haemogregarina parvula* Dias, 1953 (Adeleorina: Haemogregarinidae) to Hemolivia (Adeleorina: Karyolysidae). Afr Zool 50:165–173

Coutinho ME (2003) Population ecology and the conservation and management of *Caiman yacare* in the Pantanal, Brazil. Dissertation, Universidade de Queensland

Crawford RLDVM (2008) Information resources on reptiles. National Agricultural Library, Maryland

Criado-Fornelio A, Martinez-Marcos A, Buling-Sarana A, Barba-Carretero JC (2003) Molecular studies on Babesia, Theileria and Hepatozoon in southern Europe. Part I. Epizootiological aspects. Vet Parasitol 113:189–201

Criado-Fornelio A, Ruas JL, Casado N, Farias NA, Soares MP, Muller G, Brumt JG, Berne ME, Buling-Sarana A, Barba-Carretero JC (2006) New molecular data on mammalian Hepatozoon species (Apicomplexa: Adeleorina) from Brazil and Spain. Int J Parasitol 92:93–99

Dariba D, Toboada GL, Doallo R, Posada D (2012) JModelTest 2: more models, new heuristics and parallel computing. Nat Methods 9:772

Davies AK, Johnston MRL (2000) The biology of some intraerithrocytic parasites of fishes, amphibian and reptiles. Adv Parasitol 45:1–107

Davies AK, Sterrett S (2011) Prevalence of haemogregarine parasites in three freshwater turtles species in a population in northeast Georgia, USA. Int J Zoo Res 7:156–163

Davies AJ, Smith NJ, Hayes PM, Seddon AM, Wertheim D (2004) *Haemogregarina bigemina* (Protozoa: Apicomplexa: Adeleorina) past, present and future. Folia Parasitol 51:99–108

Demoner LC, Magro NM, Da Silva MR, De Paula Antunes JM, Calabuig CI, O'Dwyer LH (2016) Hepatozoon spp. infections in wild rodents in an area of endemic canine hepatozoonosis in southeastern Brazil Ticks. Tick Borne Dis 7:859–864

Dvoráková N, Kvicerová J, Papoušek I, Javanbakht H, Tiar G, Kami H, Široký P (2014) Haemogregarines from western Palaearctic freshwater turtles (genera *Emys, Mauremys*) are conspecific with *Haemogregarina stepanowi* Danilewsky, 1885. Parasitology. 141(4):522–30

Dvoráková N, Kvicerová J, Hostovský M, Siroký P (2015) Haemogregarines of freshwater turtles from Southeast Asia with a description of *Haemogregarina sacaliae* sp. n. and a redescription of *Haemogregarina pellegrini* Laveran and Pettit, 1910. J Parasitol 142:816–826

Eisen RJ, Schall JJ (2000) Life history of malaria parasite (*Plasmodium mexicanum*): independent traits and basis for variation. Proc Biol Sci 267:793–799

Ferrari AS (1980) Quelônios: animais em extinção. Manaus, Falangola

Garcia-Navarro CEK, Pachalli JR (1994) Manual de hematologia veterinária. Livraria Varela, São Paulo

Godfrey RD Jr, Fedynich AM, Pence DB (1987) Quantification of haemotozoa in blood smears. J Wildlife Dis 23:558–565

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59:307–321

Haklová B, Majláthová V, Majláth I, Harris DJ, Petrilla V, Litschka-Koen T, Oros M, Petko B (2013) Phylogenetic relationship of *Hepatozoon* blood parasites found in snakes from Africa, America and Asia. Parasitology 141:389–398

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Ac Symp Series 41:95–98

Ibama – Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (1989) Projeto Quelônios da Amazônia—10 anos. IBAMA, Brasília

Jakes KA, O'Donoghue P, Munro M, Adlard R (2001) Hemoprotozoa of fresh turtles in Queensland. J Wildl Dis 37:12–19

Kopecna J, Jirku M, Obornik M, Tokarev YS, Lukes J, Modry D (2006) Phylogenetic analysis of coccidian parasites from invertebrates: search for missing links. Protist 157:173–183

Kvičerová J, Pakandl M, Hypša V (2008) Phylogenetic relationships among Eimeria spp. (Apicomplexa, Eimeriidae) infecting rabbits: evolutionary significance of biological and morphological features. Parasitol 135:443–452

Kvicerova J, Hypsa V, Dvorakova N, Mikulicek P, Jandzik D, Gardner MG, Javanbakht H, Tiar G (2014) Siroky P. H emolivia and Hepatozoon: Haemogregarines with Tangled Evolutionary Relationships Protist 165:688–700

Lainson R, Naiff R (1998) *Haemoproteus* (Apicomplexa: Haemoproteidae) of tortoises and turtles. Proc R Soc B 265:941–949

Levine ND (1988) The protozoan phylum Apicomplexa. CRC Press, Boca Raton

Maia JP, Harris DJ, Perera A (2011) Molecular survey of Hepatozoon Species in Lizards From North Africa. J Parasitol 97:513–517

Maia JP, Pereira A, Harris DJ (2012) Molecular survey and microscopic examination of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina) in lacertid lizards from western Meditarranean. Folia Parasitol 59:241–248

Maia JP, Harris DJ, Carranza S, Gómez-Días E (2014) A comparison of methods for estimating parasitemia of hemoparasites (Apicomplexa: Adeleorina) and its application for studying infection in natural populations. PLoS One 9:1–10

Maia JP, Harris DJ, Carranza S, Goméz-Días E (2016) Assessing the diversity, host-specificity and infection patterns of apicomplexan parasites in reptiles from Oman, Arabia. Parasitology 143:1730–1747

Malvasio A, Souza AM, Gomes N, Sampaio FAA, Molina FB (2002) Variações ontogenéticas na morfometria e morfologia do canal alimentar pós-faríngeo de *Trachemys dorbignyi* (Duméril e Bibron, 1835) *Podocnemis expansa* (Schweigger, 1812), *P. unifilis* (Troschel, 1848) *P. sexturberculata* (Cornalia, 1849) (Anapsida: Testudines). PblAvul Inst Pau Bras His Nat 5:39–51

Mathew JS, Van Den Bussche RA, Ewing SA, Malayer JR, Latha BR, Panciera RJ (2000) Phylogenetic relationships of Hepatozoon (Apicomplexa: Adeleorina) based on molecular, morphologic, and lifecycle characters. J Parasitol 86:366–372

McAllister CT, Bursey CR, Robison HW, Connior MB, Barger MA (2014) *Haemogregarina* sp. (Apicomplexa: Haemogregarinidae) *Telorchis attenuate* (Digenea: Telorchiidae) and *Neoechinorhynchus mauydis* (Acanthocephala: Neoechinorhynchidae) form map turtles (*Graptemys* spp.), in northcentral Arkansas. J Ark Acad Sci 68:154–157

McCoy JC, Failey E, Price SJ, Dorcas ME (2007) An assessment of leech parasitism on semi-aquatic turtles in the Eastern Piedmont of North Carolina. Southeast Nat 6:191–202

Mihalca AD, Achelaripei D, Popescu P (2002) Haemoparasites to the genus *Haemogregarina* in a population of European pond turtles (*Emys orbicularis*) from Dragasani, Valcea country, Romenia. Rev Sci Parasitol 3:22–27

Moço TC, O'Dwyer LH, Vilela FC, Barella TH, Silva RJ (2002) Morphologic and morphometric analysis of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) of snakes. Mem Inst Oswaldo Cruz 97:1169–1176

Moço TC, Silva RJ, Madeira NG, Dos Santos PK, Rubini AS, Leal DD, O'Dwyer LH (2012) Prevalência de *Hepatozoon* spp. em serpentes e caracterização morfológica, morfométrica e molecular de *Hepatozoon* (Apicomplexa, Hepatozoidae) de *Crotalus durissus terrificus* (Serpentes, Viperidae) naturalmente infectadas. Parasitol Res 110:1393–1401

Møller AP (1997) Parasitism and the evolution of host life history. In: Clayton DH, Moore J (eds) Host–parasite evolution: general principals and avian models. Oxford University Press, Oxford, pp 105–127

O'Dwyer LH, Moço TC, Paduan S, Spenassatto C, Silva RJ, Ribolla PE (2013) Description of three new species of Hepatozoon (Apicomplexa, Hepatozoidae) from rattlesnakes (*Crotalus durissus terrificus*) based on molecular, morphometric and morphologic characters. Exp Parasitol 135:200–207

Olsson M, Wapstra E, Madsen T, Ujvari B, Rugfelt C (2005) Costly parasite resistance: a genotype-dependent handicap in sand lizards? Biol Lett 1:375–377

Oppliger A, Clobert J (1997) Reduced tail regeneration in the common lizard, Lacerta vivipara, parasite by blood parasites. Fund Ecol 11: 652–655

Oppliger A, Célérier ML, Clobert J (1996) Physiological and behavioral changes in common lizards parasite by haemogregarines. Parasitology 133:433–438

Oyamada M, Davoust B, Boni M, Dereure J, Bucheton B, Hammad A, Itamoto K, Okuda M, Inokuma H (2005) Detection of Babesia canis rossi, B. canis vogeli, and Hepatozoon canis in dogs in a village of eastern Sudan by using a screening PCR and sequencing methodologies. Clin Diagn Lab Immunol 12:1343–1346

Ozvegy J, Marinkovic D, Vucicevic M, Gajic B, Stevanovic J, Marinkovic D, Stevanovic J, Krnjaic D, Aleksic Kovacevic S (2015) Cytological and molecular identification of *Haemogregarina stepanowi* in blood samples of the European pond turtle (*Emys orbicularis*) from quarantine at Belgrade zoo. Acta Vet 65:443–453

Perkins SL, Keller AK (2001) Phylogeny of nuclear small subunit rRNA genes of haemogregarines amplified with specific primers. J Parasitol 87:870–876

Pessoa LMB, Scalon MC, Carneiro FT, Lima MGM, Silva TF, Monteiro RV, Paludo GR (2016) Ocorrência de hemogregarina em duas espécies de cágados brasileiros. Acta Sci Vet 44:1381

Picelli AM, Carvalho AV, Viana LA, Malvasio A (2015) Prevalence and parasitemia of *Haemogregarina* sp. in *Podocnemis expansa*

(Testudines: Podocnemididae) from the Brazilian Amazon. Parasitol Res 113:4499–4503

Portelinha TCG, Malvasio A, Piña CI, Bertoluci JA (2013) Reproductive allometry of *Podocnemis expansa* (Testudines: Podocnemididae) in southern Brazilian Amazon. J Herpetol 47:232–236

Readel AM, Philliphs CA, Wetzel MJ (2008) Leech parasitism in a turtle assemblage: effects of hot and environmental characteristics. Copeia 2008(1):227–233

Salera-Junior G, Malvasio A (2005) Biologia e Conservação dos Quelônios da Amazônia. Universidade Federal do Tocantins, Palmas

Sandland GJ, Minchella DJ (2003) Costs of immune defense: an enigma wrapped in an environmental cloack? Trend Parasitol 19:571–574

Sehgal RN, Jones HI, Smith TB (2005) Blood parasites of some west African rainforest birds. J Vet Med Sci 67:295–301

Siddal ME, Desser SS (1993) Ultrastructure of merogonic development of *Haemogregarina* (sensulato) *myoxocephali* (Apicomplexa: Adeleorina) in the marine leech *Malmiana scorpii* and localization of infective stages in the salivary cells. Eur J Protistol 29:191–201

Siddall ME (1995) Phylogeny of Adeleid blood parasites with a partial systematic revision of the haemogregarine complex. J Eukaryot Microbiol 24:1161–1125

Siddall ME, Desser SS (1991) Merogonic development of *Haemogregarina balli* (Apicomplexa: Adeleorina: Haemogregarinidae) in the leech *Placobdella ornate* (Glossiphoniidae), its transmission to a chelonian intermediate host and phylogenetic implications. J Parasitol 77:426–436

Siddall ME, Desser SS (1992) Prevalence and intensity of *Haemogregarinaballi* (Apicomplexa: Adeleorina: Haemogregarinidae) in three turtles species from Ontario, with observations on intraerythrocytic development. Can J Zool 70:123–128

Siddall ME, Desser SS (2001) Transmission of *Haemogregarina balli* from painted turtles to snapping turtles through the leech *Plocobdella ornate*. J Parasitol 87:1217–1218

Siroký P, Kamler M, Modrý D (2004) Long-term occurrence of *Hemolivia* cf. *mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in captive *Testudo marginata* (Reptilia: Tetsudinidae): evidence for cyclic merogony? J Parasitol 90:1391–1393

Sloboda M, Kamler M, Bulantova J, Votypka J, Modry D (2007) A new species of Hepatozoon (Apicomplexa: Adeleorina) from Python regius (Serpentes: Pythonidae) and its experimental transmission by a mosquito vector. J Parasitol 93:1189–1198

Soares P, Brito ES, Paiva F, Pavan D, Viana LA (2014) *Haemogregarina* spp. in wild population from *Podocnemis unifilis* Trschel, 1848 in the Brazilian Amazon. Braz J Vet Parasitol 24:191–197

Telford SR Jr (1971) Parasitic diseases of reptiles. J Am Vet Med Assoc 159:1644–1652

Telford SR Jr (2009) Hemoparasites of the reptilian: color atlas and text. CRC Press, Boca Raton

Thrall PH, Hochberg ME, Burdon JJ, Bever JD (2007) Coevolution of symbiotic mutualists and parasites in a community context. Trend Eco Evol 22:120–126

Ujvari B, Marques EJ (2005) High prevalence of *Hepatozoon* spp. (Apicomplexa: Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical Australia. J Parasitol 90:670–672

Vogt RC (2008) Tartarugas da Amazônia. Biblos, Lima

Wosniak EJ, Mclaughlin GL, Telford SR (1994) Description of the vertebrate stages of a hemogregarine species naturally infecting Mojave Desert sidewinders (*Crotalus cerastes cerastes*). J Zoo Wildl Med 25:103–110

Zippel KC, Lillywhite HB, Mladinich CRJ (2001) New vascular system in reptiles: anatomy and postural hemodynamics of the vertebral venous plexus in snakes. J Morphol 250:173–184