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Research article

Host species and age-specific variation on *Hepatozoon* prevalence and its effect on body condition in two Neotropical crocodiles

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Many populations of species belonging to the order Crocodilia are threatened due to illegal trafficking, indiscriminate hunting, and habitat loss and degradation affecting crocodilian health and parasitic load. Although several studies have revealed that crocodiles, caimans, and alligators are frequently infected by *Hepatozoon* spp., the results from studies exploring the costs of these apicomplexan parasites on the health of their reptilian hosts are still scarce and with inconclusive results. Here, we molecularly assessed the prevalence and genetic diversity of *Hepatozoon* spp. to explore their possible influence on body condition in captive individuals of two species of Neotropical crocodilians with conservation threats, the spectacled caiman *Caiman crocodilus* and the American crocodile *Crocodylus acutus*. Fourteen percent of spectacled caimans were infected by *H. caimani*, whereas no American crocodiles showed infection. The prevalence of *Hepatozoon* in spectacled alligators varied along age, where subadult individuals were the most frequently parasitized. Surprisingly, the body condition of infected individuals was significantly higher than body condition of uninfected spectacled caimans, which suggests greater negative effects of the infection in individuals of poor quality. Also, the body condition of subadult individuals was significantly higher than body condition of juveniles of both alligator species, likely reflecting differences in the occupancy of habitats with higher resource abundance, or variations in the nutritional

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values of the diet between these age classes. These outcomes provide valuable information on disease ecology for developing conservation strategies and the management conservation of wildlife populations of these species.

Keywords: blood parasites, body condition, *Caiman crocodilus*, *Crocodilus acutus*, *Hepatozoon caimani*, wildlife conservation.

Introduction

Haemogregarines are apicomplexan blood parasites that commonly infect all groups of vertebrates. These intracellular parasites show an obligatory heteroxenous life cycle, where asexual reproduction occurs in the vertebrate host, while sexual multiplication occurs in the hematophagous invertebrate vector (Telford 2009). They are the most prevalent parasites in reptiles (Smith 1996). Currently, four genera of Haemogregarines are known to parasite reptiles: *Karyolysus*, *Hemolivia*, *Haemogregarina*, and *Hepatozoon* (Smith 1996, Telford 2009). Among them, the *Hepatozoon* species are the most common and more widely distributed Haemogregarines in reptiles (Telford 1984, 2009). However, despite the high occurrence and diversity of these parasites among the four major groups of Reptilia (Amphisbaenia, Chelonia, Crocodilia, Serpentes), a large number of new blood apicomplexan species is yet to be discovered in this group of vertebrates (Duszynski 2021).

Moreover, the pathogenicity of infection by these parasites is unclear, and the results of studies analyzing their potential negative effects on their reptilian hosts have revealed mixed results. On one hand, several studies have shown that *Hepatozoon* infections can be harmful for their reptilian hosts. For example, Knotkova et al. (2005) reported anaemia, low hemoglobin, basophilia, eosinophilia, heterophilia, and azurophilia in Malaysian giant turtles *Orlitia borneensis* infected with intraerythrocytic haemogregarines. In addition, Miyamoto and Mello (2007) analyzed whether the infection by *Hepatozoon* spp. affected the incidence of DNA fragmentation and cell death in red blood cells from the rattlesnake *Crotalus durissus terrificus*, showing that the parasite infection was associated with an accelerated destruction of erythrocytes in the reptile host, which may provoke anaemia in these individuals. Also, Wozniak et al. (1996) experimentally infected three lizard species *Sceloporus undulatus*, *Eumeces obsoletus,* and *Sceloporus poinsetti* with *Hepatozoon mocassini* from a cotton-mouth moccasin *Agkistrodon piscivorus leucostoma*, showing severe lethargy, anorexia, leukocytosis, and multifocal random hepatocellular necrosis in all three lizard species.

On the other hand, other studies have failed to report adverse effects associated with *Hepatozoon* infection in reptiles. For example, Brown et al. (2006) did not find any negative effect on host fitness (body condition, growth rate, feeding rate, antipredator behaviour, locomotor performance, reproductive status, reproductive output, and recapture rate) in keelback snakes *Tropidonophis mairii* heavily infected with haemogregarine blood parasites. Similarly, Damas-Moreira et al. (2014) estimated the effect of *Hepatozoon* infection on the flight-initiation distance from a simulated predator in the Andalusian wall lizard *Podarcis vaucheri*. They showed that the escape distance was not associated with *Hepatozoon* parasitemia, hence suggesting a limited impact of the parasite infection on their hosts. Also, Marzal et al. (2017) assessed the effects of *Hepatozoon* infection on the Spanish terrapins *Mauremys leprosa* and found no differences in body size and body condition among infected and uninfected individuals. Moreover, the effects of Haemogregarines on body condition can vary enormously between related species of lizards, even between species within the same genus. For example, Amo et al. (2004) showed that haemogregarine parasitemia had a negative effect on the body condition during the reproductive season in the Iberian rock lizard *Lacerta monticola*. However, haemogregarine infection was not related to body condition in the ocellated lizard *L. lepida* (Amo et al. 2005).

The order Crocodilia (suborder Eusuchia) consists of 27 species of alligators, caimans, crocodiles, and gharials with an almost cosmopolitan distribution, which inhabit tropical and subtropical areas (Stevenson 2019). Beyond the potential threat posed by infectious diseases (see above), many populations of these reptiles are threatened due to the alteration and destruction of their habitat, indiscriminate hunting, and illegal trafficking of species (Ponce-Campos et al. 2012). In fact, seven crocodilian species are critically endangered, four are vulnerable, and the remaining twelve are considered as low risk, although updated information on these species is needed (IUCN 2021). In a recent review, it was shown that 63% of crocodilian species are frequently infected by apicomplexan blood parasites, being most diverse and abundant in the genus *Hepatozoon* (Duszynski et al. 2020). In fact, several species of *Hepatozoon* have been found infecting crocodilians worldwide, such as *H. serrei* in the smooth-fronted caiman *Paleosuchus trigonatus* (Smith 1996), *H. crocodilinorum* in the American alligator *Alligator mississippiensis* (Davis et al. 2011), *H. pettiti* in the Nile crocodile *Crocodylus niloticus* (Leslie et al. 2011) and in the marine crocodile *C. porosus* (Duszynski et al. 2020), *H. sheppardi* in the Nile crocodile (Santos Dias 1952), and *H. caimani* in the overo caiman *Caiman latirostris* (Smith 1996), the alligator *C. yacare* (Viana et al. 2010a, Soares et al. 2017a), and the spectacled caiman *C. crocodilus* (Lainson et al. 2003, Soares et al. 2017b). However, despite the increasing number of descriptive studies of the prevalence, morphology, diversity, and phylogenetic relationships of the parasites of the genus *Hepatozoon* in crocodiles, caimans, and alligators (Bouer et al. 2017, Duszynski et al. 2020), the potential negative effects of these parasites on the biological fitness of this group of reptiles have been poorly analyzed. The few studies carried out have found hardly any detrimental effects on their hosts, suggesting that the parasites of the genus *Hepatozoon* are of low or null pathogenicity in crocodilians (Lovely et al. 2007, Leslie et al. 2011, Soares et al. 2017a). Considering the threats to the survival of many populations of crocodilians, the study of any potential source of mortality or stress, such as these blood parasites, should be a priority for its conservation.

Captive breeding facilities, rehabilitation centers, zoos, and other facilities for captive animal management play a key role in species conservation. For example, they provide opportunities for captive breeding programs, and supplementing natural populations (Ziegler et al. 2022). Also, the study of parasites in amphibians and reptiles held or bred in captivity is essential in release programs because they can influence the translocation success or transmit new parasites to wildlife populations (Germano and Bishop 2009, Beckmann et al. 2022). For example, recent investigations in captive Orinoco crocodiles *Crocodylus intermedius*, the most threatened crocodilian of South America (Balaguera-Reina et al. 2018a), have enabled the deciphering of novel and baseline data for haematological and biochemistry values with special importance for the clinical diagnosis and wildlife disease ecology (Barajas-Valero et al. 2021), as well as the evaluation of the genetic characterization determining the viability of reintroduction programs from ex situ populations of this critically endangered species (Saldarriaga-Gomez et al. 2023).

In this study, we analyzed the relationship between infection by *Hepatozoon* spp. and body condition, a proxy of health state, in individuals of various age classes of two species of Neotropical crocodilians with conservation threats, the spectacled caiman *Caiman crocodilus* and the American crocodile *Crocodylus acutus*. We used molecular methods for the detection of *Hepatozoon* parasites in blood samples from individuals of these two species maintained in several fauna recovery centers of the Peruvian Amazon and one aquaculture center from northeast Peru. We predicted that, if *Hepatozoon* infection were harmful, this would be reflected in a lower body condition of parasitized individuals.

Material and methods

Study species

The spectacled caiman *Caiman crocodilus*, also known in Peru as common caiman or white caiman, is the alligator with the widest distribution in the Neotropics, inhabiting different types of freshwater courses and swamps throughout the American intertropical belt, from Mexico to the center of Brazil (Balaguera-Reina and Velasco 2019). This species has been exploited for local food and for commercial purposes, both for national and international trade (Caldwel 2017). For this reason, different specific protocols for its conservation have recently started in several South American countries (Velasco and Balaguera-Reina 2018).

The American crocodile *Crocodylus acutus*, also named the Tumbes crocodile in Peru, has a wide distribution on both coasts of the Neotropical area of the American continent, from Sinaloa (Mexico) to Peru on the Pacific coast, and from the extreme south of Florida to the plains of the Orinoco (northeast Venezuela) in the Atlantic, including Cuba, Jamaica, and Haiti (Ernst et al. 1999, Thorbjarnarson 2010). It is a large crocodile (up to 6 m in length), being the second largest crocodile in America. Despite its wide distribution, it is an endangered species listed as vulnerable by the International Union for the Conservation of Nature (IUCN) due to the population decline caused by habitat destruction and loss, and poaching (Ponce-Campos et al. 2012). Importantly, this species is listed as critically endangered in Peru because of loss of habitat and massive hunting (SERFOR 2018).

Study sites

Thirty-five spectacled caimans were seized from illegal wildlife trade by Autoridad Regional Ambiental (ARA) and Servicio Nacional Forestal y de Fauna Silvestre (SERFOR) of the San Martín and Loreto regions (Peru) and were transported and housed in three rescue centers after seizure. Twenty-eight *C. crocodilus* (21 juveniles and seven neonates) were collected in the Amazon Rescue Center (CREA) (3°53′0″S, 73°21′4″W) in the Loreto region, located at km 4.5 of the road Iquitos–Nauta, southwest of the city of Iquitos. Juvenile *C. crocodilus* were housed in individual 3 × 5 m pools of 1.5 m height and a water depth of 0.5 m with several artificial islands. The smaller specimens (neonates) were housed in similar enclosures in groups of 3–5 individuals. In addition, five *C. crocodilus* (four subadults and one adult) were collected in Selva Viva, located 11.6 km from the center of Tarapoto (6°26′54″S, 76°28′19″W). Individuals were housed in a large, enclosed area with a surface area of 40 × 100 m and a large central lagoon 1 m deep that occupied 80% of the area. Also, two *C. crocodilus* (one juvenile and one subadult) were collected in Chullachaqui, located 15.5 km distance from the center of the city of Tarapoto on the road to Yurimaguas (6°27′49″S, 76°19′3″W). In this center, caimans were individually housed in 15 m^2 wooden pens with a central pool of 0.5 m depth to allow immersion. All spectacled caimans had been housed in these facilities between 1–4 months before we carried out blood sampling.

American crocodiles ($n=68$: one neonate, 19 juveniles, 34 subadults, and 14 adults) were sampled at Tuna Carranza aquaculture center (3°30′36″S, 80°23′46″W), an experimental *C. acutus* hatchery for repopulation purposes belonging to the National Fisheries Development Fund (FONDEPES), located in the district and province of Tumbes in the Tumbes Region. All American crocodiles had been raised in captivity and kept in large enclosures with water pools, according to the recommendations from Ziegler (2001) for enclosure sizes for captive crocodiles. The specimens were separated by age in 15 m × 12 m enclosures, and each pen had a 1 m deep central pool to allow bathing. Neonates and juveniles were housed in two enclosures with ten individuals in each, whereas subadults and adults of *C. acutus* were housed separately with a maximum of 11 individuals in each enclosure. All animals were sampled in June 2017, apart from the *C. crocodilus* at CREA, which were sampled again in June 2018.

Handling procedure, sex determination, age, body size, and body condition

The crocodiles were restrained and immobilized manually with the help of protocol knots (Rueda-Almonacid et al. 2007, Orjuela 2009). Once a crocodile was immobilized out of the water, a wet towel was placed over its eyes and it was held firmly to restrict its movement and minimize stress (Choperena and Ceballos 2016, Dodd 2016). Crocodiles were individually identified with marks on the edge of the dorsal tail plates following the codification proposed by Bolton (1989) (García-Grajales et al. 2011).

Sex was determined by exploring the cloaca using a rhinoscope to separate the edges of the cloaca and verify the genitalia by digital palpation (Webb et al. 1984, Ziegler and Olbort 2007). Age was estimated according to the body size based on previous studies. We established four categories in the case of *C. crocodilus*: neonate (< 50 cm), juvenile (51–120 cm), subadult (121–180 cm), and adult (> 181 cm) (Ayarzagüena 1983, Guerra-Cárdenas et al. 2020). We used five age categories for *C. acutus*: neonate (< 60 cm), juvenile I (61–120 cm), juvenile II (121–180 cm), subadult (181–240 cm), and adult (> 241 cm) (Lander 2003, Pérez and Escobero-Galván 2007, Seijas 2011).

Individual body condition was estimated by calculation of the Fulton body condition factor or Fulton index (K) (Ricker 1975). An assumption of this index is that the weight of a crocodile is proportional to the cube of its length. This index has been widely used to describe the physical state of numerous vertebrates (Hayes and Shonkwiler 2001) including several species of crocodiles, such as the swamp crocodile (Cedeño-Vázquez et al. 2011, Mazzotti et al. 2012), American alligator (Zweig et al. 2014), and spectacled caiman (Grant et al. 2013, Barão-Nóbrega et al. 2018). We measured the total length (TL) of individuals (from the tip of the snout to the end of the tail) using a measuring tape (accuracy ± 1 mm), and the body mass (W) with a digital scale (precision of ± 10 g). Fulton index (K) was calculated following the formula: $K = W/TL^3 \times 10^4$ (Barão-Nóbrega et al. 2018, Briggs-González et al. 2021).

Blood sampling

Blood samples were collected within a maximum period of 5 min after capture to minimize the stress response caused by handling and blood sampling (Guillette et al. 1997), immediately after physical immobilization and before the measuring and weighting procedures described above. Before sampling, we cleaned the puncture skin area with 96% ethanol-soaked cotton wool. Approximately 1 ml of blood was withdrawn from the coccygeal vein, located in the caudal sinus of the tail base, or from post-occipital spinal venous sinus (Jacobson 1993). We used 1- or 5-ml plastic syringes with heparinized disposable needles with a variable gauge, from 21G to 27G, depending on the size of the individual (Zhang 2011). After the extraction, the puncture area was pressed with cotton with ethanol until checking that there was no bleeding. Blood collected was stored in a vial with 500 µl of SET buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH = 8.0) (Sambrook et al. 1989) at room temperature for subsequent molecular analyses. All individuals were returned to their enclosures in perfect condition in less than 10 min and controlled by the staff of each center in the following days to ensure that they were in a good state of health.

Molecular detection of blood parasite infections

Genomic DNA was extracted from blood samples using the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific Inc., ref. K0722) according to the manufacturer instructions. Diluted genomic DNA (25 ng μl^{-1}) was used as template to amplify the 18S RNA gene of *Hepatozoon* spp. as described previously (Harris et al. 2011). A nested PCR reaction with primers HEMO1 and HEMO2 (Perkins and Keller 2001) for the firsrt PCR, and then primers HepF300 and HepR900 (Ujvari et al. 2004) for the second one, were used for *Hepatozoon* amplification. The amplification was evaluated by running 2.5 µl of the final PCR product on a 2% agarose gel. To verify that the PCR process was correct, we added one negative control every eight samples and two positive controls every 42 samples. Parasites detected by a positive amplification were sequenced using the procedures described by Bensch et al. (2000). Amplified fragments were sequenced from 5'end with HepF300 *Hepatozoon* spp. The obtained sequences of 600 bp were edited, aligned, and compared in a sequence identity matrix using the program MEGA (Stecher et al. 2020). A BLAST search was performed to match the product sequence to that of a known haemoparasite species.

Statistical analysis

The normality of the data distribution of all continuous variables used in statistic models was assessed with Shapiro–Wilk tests. We used a chi-square test to test for differences in the prevalence of parasites between crocodile species. Also, in the case of *C. crocodilus* (the only species in which parasites were found; see Results), a logistic regression analysis was used to explore whether sex, age, Fulton index (K factor), year of study, location of sampling, and the interaction between age and Fulton index (K factor) influenced *Hepatozoon* infection probability (infected/uninfected). A backward stepwise procedure was used to eliminate all non-significant terms ($p > 0.05$) from our starting maximal model. We used a stepwise backward procedure in a generalized linear model (GLM) with a normal distribution and an identity link function to investigate the effect of age, year of study, location of sampling, and *Hepatozoon* infection (uninfected or infected) on the body condition of *C. crocodilus*. We also used a stepwise backward procedure in a GLM with a normal distribution and an identity link function to explore the effect of age and sex on the body condition of *C. acutus*. There were no significant correlations among the predictor variables in any model (Pearson correlation test: all p-values > 0.05).

On each GLM, the stepwise procedures started with a full model including all predictors. At each step, the least significant predictor (based on the highest p-value) was removed from the model until a final model was reached. Only the final models were presented. The adequacy of the models was assessed by examining their explained variances. All analyses were performed using PASW Statistics 22 statistical package for Windows.

Results

Differences in *Hepatozoon* infection between crocodile species

We found significant differences in the prevalence of infection of *Hepatozoon* between the two species (chi square test: $\chi^2=10.210$; df = 1; $p=0.001$). Five out of the 35 *C. crocodilus* were infected with *Hepatozoon* (prevalence of infection = 14.28%), while we did not find any *C. acutus* infected by *Hepatozoon*.

Differences in *Hepatozoon* infection in relation to age of individuals, sex, body condition, year of study, and sampling location

We analysed *Hepatozoon* infection in relation to sex, age, body condition (Fulton index), year of study, and location of sampling in *C. crocodilus*. The prevalence of *Hepatozoon* varied significantly among locations of sampling (Table 1). Two and three crocodiles were infected in CREA (prevalence of infection = 7.1%; n = 28) and Selva Viva (prevalence of infection = 60%; n = 5), respectively, while no *C. crocodilus* was infected by *Hepatozoon* in Chullachaqui. We also found differences in the prevalence of *Hepatozoon* in relation to age of individuals (Table 1). Specifically, three out of five subadults were infected (60%), as were two out of 22 juveniles (9.1%). In contrast, no neonate or adult were infected with *Hepatozoon*. Moreover, the body condition of crocodiles also explained variation in the probability of infection with *Hepatozoon* (Table 1). Remarkably, infected individuals had significantly higher body condition (n = 5; mean body condition ± SD = 4.527 ± 0.807) than uninfected ones (n = 30, mean body condition ± SD = 3.323 ± 0.732) (Fig. 1). Finally, neither sex nor the interaction between body condition (Fulton index) and age significantly influenced the probability of infection with *Hepatozoon* in *C. crocodilus* (Table 1).

Genetic diversity of *Hepatozoon* lineages

We compared the parasite sequences obtained from our samples with homologous sequences of other parasite haplotypes obtained from GenBank. The obtained 18S rRNA *Hepatozoon* sequences showed 100% identity with *Hepatozoon caimani* isolates previously deposited in GenBank from *C. crocodilus* from Brazil (MF435048, MF322528, MF322539, KJ413132) and Colombia (MW246123).

Body condition versus *Hepatozoon* infection, age, and years of study

We analysed the effects of the infection by *Hepatozoon* in *C. crocodilus*. GLM analyses showed that *Hepatozoon* infection and age were related with body condition of crocodiles (Table 2). Specifically, infected individuals had significantly higher body condition than uninfected crocodiles (Fig. 1). Also, body condition from neonates was significantly lower (n = 7, mean body condition ± SD = 2.64 ± 0.45) than the body condition from juveniles (n = 22, mean body condition ± SD = 3.48 ± 0.65), subadults (n = 5, mean body condition ± SD = 4.76 ± 0.56), and adult crocodiles (n = 1, mean body condition = 3.49) (Bonferroni post hoc test: $p < 0.010$ in all cases). Similarly, the body condition of subadult crocodiles was higher than body condition from neonate, juvenile, and adult crocodiles (Bonferroni post hoc test: $p < 0.010$ in all cases) (Fig. 2). Neither the year of study nor location of sampling significantly affected body condition in *C. crocodilus* (Table 2).

Finally, we found that body condition varied with the age of individuals in *C. acutus* (Table 3). Specifically, body condition of subadult crocodiles was significantly higher than body condition of individuals from other age classes (subadults: n = 34, mean body condition ± SD = 5.38 ± 1.25; juveniles I: n = 1, mean body condition = 3.78; juveniles II: n = 19, mean body condition ± SD = 3.88 ± 0.63; adults: n = 14, mean body condition ± SD = 4.46 ± 0.74) (Bonferroni post hoc test: all $p < 0.010$) (Fig. 3). Male and female American crocodiles did not differ in body condition (Table 2) (males: n = 53, mean body condition ± SD = 4.72 ± 1.14; females: n = 12, mean body condition ± SD = 4.85 ± 1.41).

Discussion

Investigations of wildlife maintained in captivity are of special relevance for endangered or poorly studied species. For

Table 1. Results from the stepwise backwards logistic regression to predict the probability of *Hepatozoon* infection in spectacled caimans *Caiman crocodilus*. Sex, age, Fulton index (K factor), year of study, location of sampling, and the interaction between age and Fulton index (K factor) were included in the analysis as predictor variables. Only independent variables selected by the consensus model are listed. Sample size was 35 individuals. Significant factors are highlighted in bold.

Independent variable	B	SE	Wald	DF	p-value	Exp (B)
Age	0.774	0.401	3.720	3	**0.047**	2.152
Location of sampling	1.126	0.552	4.157	2	**0.041**	3.082
Fulton index (K factor)	0.399	0.147	7.333	1	**0.007**	1.491
Constant	1.632	8.362	0.038	1	0.845	5.116

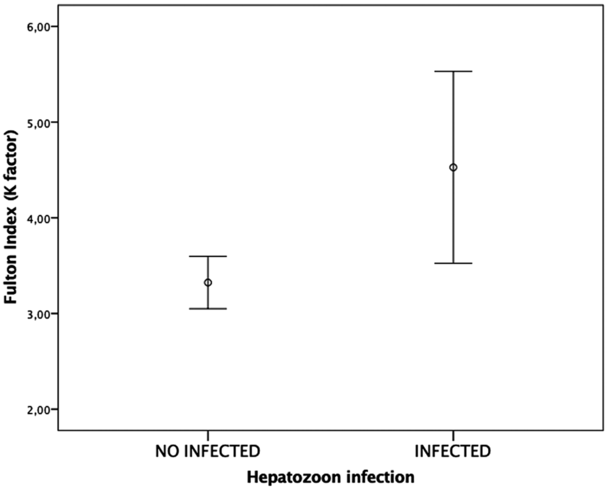

Figure 1. Barplots with error bars showing Fulton index (K factor) as an estimate of body condition for infected (n = 5) and uninfected (n = 30) spectacled caimans *Caiman crocodilus*. Error bars plots show means ± 95% of confidence interval.

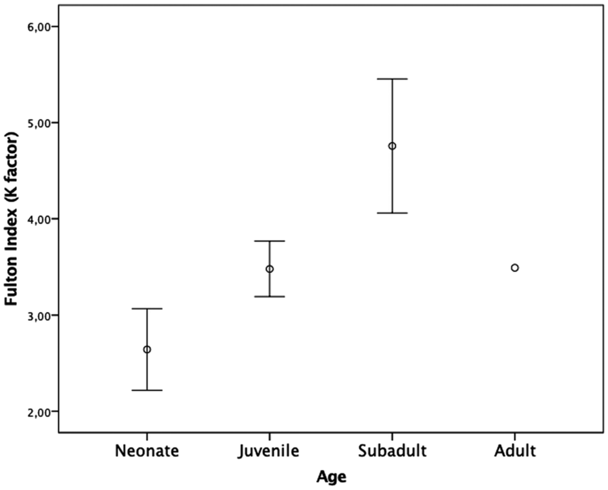

Figure 2. Barplots with error bars showing Fulton index (K factor) as an estimate of body condition for neonate (n = 7), juvenile (n = 22), subadult (n = 5) and adult (n = 1) spectacled caimans *Caiman crocodilus*. Error bars plots show means ± 95% of confidence interval.

example, they have provided valuable information about the life cycle of the parasite (Valkiūnas 2004, Jacobson 2007), the negative impact of the parasite infection on the health of their hosts (Valkiūnas 2004, Jacobson 2007, Santiago-Alarcon and Marzal 2020), and the susceptibility of the host to become infected by a wide range of parasites (Mewius et al. 2021). Furthermore, wildlife birds and reptiles kept in captivity are an excellent model for parasite research and provide valuable results for wildlife management conservation. In this sense, the development of many anti-parasite drugs and chemotherapies has been possible thanks to the use of individuals from zoos and rehabilitation centers (Kent 2004, Palinauskas et al. 2020). Here, we explored the relationships between body condition and *Hepatozoon* infection in two species of Neotropical crocodiles. The main results were: 1) about 15% of *C. crocodilus* were infected with *H. caimani*, whereas no *C. acutus* were infected with *Hepatozoon*; 2) age, body condition, and sampling location explained variations in the probability of *Hepatozoon* infection in *C. crocodilus*; 3) *C. crocodilus* infected with *Hepatozoon* showed higher body condition than uninfected individuals; and 4) body condition varied significantly among age categories in both crocodile species, where subadult individuals showed higher body condition.

Table 2. Results from the generalized linear model (GLM) explaining variation in Fulton index (K factor) as an estimate of body condition for spectacled caimans *Caiman crocodilus*. A backward stepwise procedure was used in the analysis with age, prevalence of *Hepatozoon* infection, year of study, and location of sampling predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 35 individuals. Significant factors are highlighted in bold.

Independent variable	Estimate	SE	t	p-value
Age	0.535	0.170	3.151	**0.001**
Hepatozoon infection	0.653	0.278	2.354	**0.028**

Prevalence and genetic diversity of *Hepatozoon* parasites

Our results showed that about 15% of *C. crocodilus* were infected with *Hepatozoon*. This prevalence of infection is lower than that found in other studies in free-living populations of this crocodile species in Brazil (80%) (Soares et al. 2017a) and Peru (90%) (Erazo and Capunay Becerra 2020), but similar (10%) to that found in captive populations in Peru (Rojas et al. 2011). Variations in the prevalence of blood parasite infection between populations are usually attributed to environmental differences that impair the abundance of vectors that transmit these parasites (Martínez-Abraín et al. 2004, Martín et al. 2016, Marzal et al. 2017). Alternatively, these differences may also be due to proper handling and low stress conditions that minimize vector presence and immunosuppression of captive animals, respectively (Derraik 2005, Manolis and Webb 2016). Although it is assumed that glucocorticoids levels, the steroid hormones released as a response to stressful challenges, are normally higher in captive animals than in their wild counterparts (Karaer et al. 2023), it has

Table 3. Results from the generalized linear model (GLM) explaining variation in Fulton index (K factor) as an estimate of body condition for American crocodiles *Crocodylus acutus*. A backward stepwise procedure was used in the analysis with age and sex of individuals as predictor variables. Only independent variables selected by the backward stepwise procedure are listed. Sample size was 68 individuals. Significant factors are highlighted in bold.

Independent variable	Estimate	SE	t	p-value
Age	0.396	0.194	2.045	**0.048**

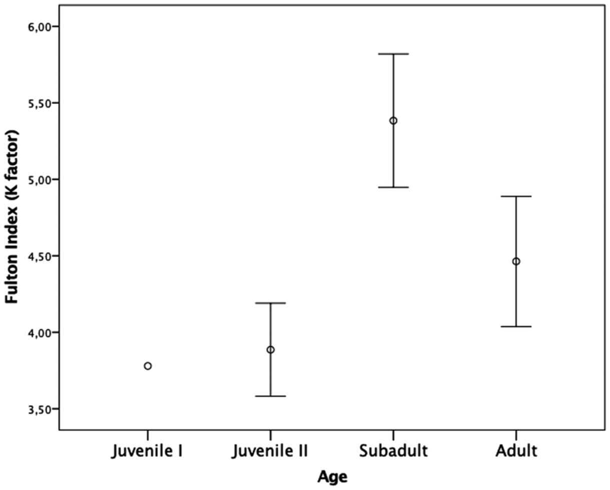

Figure 3. Barplots with error bars showing Fulton index (K factor) as an estimate of body condition for juvenile I (n = 1), juvenile II (n = 19), subadult (n = 34), and adult (n = 14) American crocodiles *Crocodylus acutus*. Error bars plots show means ± 95% of confidence interval.

recently been reported that captive and wild American alligators show similar values of measurements to assess stress levels (i.e. corticosterone levels and heterophil/lymphocyte ratios) (Merchant et al. 2023), indicating that crocodiles maintained in good captive environments may experience similar chronic stress to wild animals. In addition, variations in blood parasite infections between populations are usually attributed to environmental differences in the abundance of vectors or density of vertebrate hosts (Grenfell and Dobson 1995). Unfortunately, given that the prepatent period of *H. caimani* in *C. crocodilus* ranges between 52 and 82 days (the time elapsed between the transmission of the parasite to the crocodile and detection of gametocytes in its blood) (Lainson et al. 2003), and because spectacled caimans were housed in these facilities between 1 and 4 months before we took blood samples, we could not assess whether *Hepatozoon* infection was acquired in their natural environment or in captivity after their seizure.

In contrast to the observed prevalence of *Hepatozoon* in *C. crocodilus*, no *C. acutus* were infected with this apicomplexan parasite. These interspecific differences in prevalence of infection may be due to habitat variations in the presence or abundance of transmitting vectors of *Hepatozoon*. *Culex* spp. mosquitoes have been identified as natural vectors of this parasite (Lainson et al. 2003, Viana et al. 2010b). However, culicids are not typically eaten by crocodilians, and hence it has been suggested that the main transmission route of *H. caimani* in South America is through the ingestion of paratenic hosts (insectivorous vertebrates such as anurans) (Viana et al. 2012, Pereira et al. 2014, Matta et al. 2022). *Caiman crocodilus* live in fresh waters in Amazonia (Balaguera-Reina and Velasco 2019), which are also common habitats for *Culex* mosquitoes (Rueda 2008). In contrast, *C. acutus* usually inhabits permanent bodies of water such as saltwater sections of rivers, coastal lagoons, and estuaries (Thorbjarnarson 2010). The waters of these habitats have higher salinity than those of rivers and other freshwater areas, which decreases the survival of the larvae of *Culex* mosquitoes, among others (Kengne et al. 2019). Therefore, the lower abundance of *Hepatozoon* transmitting vectors in coastal and brackish areas would decrease the abundance of infection in potential paratenic hosts such as anurans and fish, which would explain the absence of infection with *H. caimani* in *C. acutus*. Nonetheless, we cannot discard the possibility that the sampled individuals were not infected simply because the conditions of the breeding center were better than in the natural environment.

Contrary to our results, a previous study based only on microscopic observations reported a prevalence of 16% of *Hepatozoon* spp. in *C. acutus* individuals from the same center where we did our study (zoocriadero Tuna Carranza) (Enríquez et al. 2014). To the best of our knowledge, there is no other microscopic or molecular study showing *Hepatozoon* infection in this species. However, it has been pointed out that the validity of microscopic identification of the genus *Hepatozoon* in many published studies is questionable because the morphological characteristics of gametocytes are similar to those of other apicomplexans, such as *Haemogregarina* (Soares et al. 2017a). Thus, it may be possible that the parasites observed by Enríquez et al. (2014) could be *Haemogregarina* parasites rather than *Hepatozoon*, because there has been confusion in assigning parasites to one or another genus in crocodilians for many years (Lainson et al. 2003, Tellez 2014, Duszynski et al. 2020). In support of this idea, *C. acutus* has been described as being infected by leeches, the main vector of *Haemogregarina* spp. (García-Grajales and Buenrostro-Silva 2011).

To date, 17 out of 27 species of Crocodilia have been examined in search for apicomplexan parasites (Duszynski 2021). *Hepatozoon caimani* was the only parasite species found in our study. This parasite species is widely distributed among crocodilians in South America, as it has been found infecting *C. latirostris* (Smith 1996, Duszynski et al. 2020), *C. crocodilus* (Lainson et al. 2003, Duszynski et al. 2020), *C. yacare* (Lainson et al. 2003, Viana et al. 2010a, Duszynski et al. 2020), and the dwarf caiman *Paleosuchus palpebrosus* (Clemente et al. 2023). Although few species of this parasite genus have been described in South American crocodilians, it is predicted that more than 30 species of apicomplexan parasites remain to be discovered from species of Crocodilia (Duszynski 2021). Therefore, further studies exploring the prevalence and diversity of blood parasites in this group of endangered vertebrates are required.

Variations in *Hepatozoon* infection in relation to age, sex, and localities of sampling

Subadult *C. crocodilus* showed a higher prevalence of infection than juveniles. These results agree with those found by Viana et al. (2010a) in *C. yacare* in Pantanal (Brazil), suggesting that caimans are first infected at juvenile age, when their diet changes from eating invertebrates to predating on anurans and fish (Viana et al. 2010a), which are paratenic hosts of *H. caimani* (Viana et al. 2012, Pereira et al. 2014).

Likewise, given the viability and persistence of *H. caimani* in their hosts (Davis and Johnston 2000), the higher prevalence of parasites in subadult individuals than in juveniles can be explained by the succession and accumulation of infections throughout the life of the alligator (Viana et al. 2010a), as has been observed in haemoparasite infections in lizards (Amo et al. 2005), snakes (Santos et al. 2005), and other species of crocodiles (Leslie et al. 2011).

We reported differences in prevalence of infection among the fauna recovery centers where *C. crocodilus* were housed and sampled. These variations are likely to have been caused by differences in the density of vertebrate hosts, as well as divergences in environmental and/or handling conditions potentially influencing the probability of *Hepatozoon* infections (Grenfell and Dobson 1998, Derraik 2005, Manolis and Webb 2016). Moreover, we cannot exclude the possibility that dietary differences between captive centers might have influenced the observed difference in infection rates among these centers.

We found no differences in the prevalence of infection between males and females. Although Leslie et al. (2011) reported a higher prevalence of infection in female Nile crocodiles, most studies in crocodiles and alligators found similar probabilities of infection in both sexes (Viana et al. 2010a, Erazo and Capunay Becerra 2020).

Body condition versus *Hepatozoon* infection

The negative effects of *Hepatozoon* infection show great variability among the hosts they infect. For example, in mammals, serious pathologies and even mortality associated with *Hepatozoon* infections have been described in coyotes *Canis latrans* (Kocan et al. 2000) and spotted hyenas *Crocuta crocuta* (East et al. 2008). In some reptile species it has been observed that *Hepatozoon* infections can cause anaemia and blood cell abnormalities, resulting in immunosuppression (Telford 1984). Furthermore, hepatocellular necrosis has also been observed in three species of lizards infected with *Hepatozoon mocassini* (Wozniak et al. 1996). Moreover, negative effects on growth, body condition, reproductive success, and survival associated with infection by these blood parasites have been described in the aquatic python *Liasis fuscus* (Madsen et al. 2005, Ujvari and Madsen 2006).

Although many surveys have shown a wide variety of apicomplexan parasites from alligators, caimans, and crocodiles, the low sample size of most of these studies prevented a complete taxonomic identification of these parasites, and failed to reveal pathogenic effects on their hosts (Duszynski et al. 2020). Despite the negative effects of *Hepatozoon* on their hosts presented above, the few studies carried out on crocodilians have shown that many species can tolerate the presence of infection and hardly suffer any pathogenic effects. For example, two studies in the Nile crocodile did not show significant differences in clinical hematological values between uninfected and *H. pettiti*-infected individuals (Lovely et al. 2007, Leslie et al. 2011). A greater length in infected adults than in uninfected individuals of the same age class has also been described in this species of crocodiles (Leslie et al. 2011). Furthermore, no histopathological changes were observed in the liver and lungs of *C. yacare* individuals infected by *H. caimani* (Soares et al. 2017a). Likewise, Viana et al. (2010a) did not find any negative effects of the infection with *H. caimani* on the body mass of *C. yacare* individuals.

This absence of negative effects has led to the belief that *Hepatozoon* parasites are not pathogenic in their crocodilian hosts (Leslie et al. 2011). In our study we found that infected individuals presented higher body condition than the uninfected ones, which could be in line with previous studies reporting an absence of negative effects of *Hepatozoon* in alligator and crocodiles. Alternatively, another possible explanation for these results would be a selective disappearance of low-quality individuals infected in the population, as has been suggested in birds (Marzal et al. 2016, Jiménez-Peñuela et al. 2019, Bichet et al. 2020), mammals (Lynsdale et al. 2020), and other reptile species (Madsen et al. 2005, Marzal et al. 2017). Following this idea, it has been shown in reptiles that individuals with lower body condition presented a lower immune response (Ujvari and Madsen 2006), which can lead to worse defences against blood parasite infections (Schmid-Hempel 2021). If hosts with lower body condition suffer higher mortality when infected, only those individuals with good body condition could have good defences that would allow them to counteract the negative effects of the infection and survive, leading to a positive relationship between infection by parasites and body condition (Budischak et al. 2018, Sánchez et al. 2018). In addition, Balaguera-Reina et al. (2023) recently assessed the relationship between body condition and the haematological and biochemical (blood) parameters in American alligators from Greater Everglades in Florida, showing that individuals in poorer body condition were likely dehydrated or had an inadequate diet, two of the main contributing factors to musculoskeletal disorders that may increase mortality in crocodilians (Bolton 1989, Nevarez 2006). However, our data should be interpreted with caution because of the low number of infected *C. crocodilus* in our study (n = 5). Further studies examining haematological and biochemical parameters are needed to complement our findings and determine the pathogenicity of *Hepatozoon* infection in *C. crocodilus*.

Body condition and age categories

The body condition varied significantly among age categories in both alligator species, where subadult *C. crocodilus* and *C. acutus* showed higher body condition than juvenile individuals. Similarly, Ojeda-Adame et al. (2020) reported that adult and subadult *C. acutus* had higher values of body condition than juvenile individuals. Also, Mazzotti et al. (2012) showed that adult swamp crocodiles (*Crocodylus moreletii*) had higher body condition than younger crocodiles. These differences are likely because adult and subadult crocodiles generally occupy habitats with better and more abundant resources than juveniles, and/or have better access to food with higher nutritional content (Mazzotti et al. 2012). Alternatively, the higher body condition in adult and subadult alligators can be explained by differences in diet between age groups, where the consumption of invertebrates (insects and crustaceans) decreases with age in favour of larger prey such as fish and mammals (Wallace and Leslie 2008, Adame et al. 2018, Balaguera-Reina et al. 2018b), whose protein contribution represents a greater nutritional value (Hernández et al. 2018).

In conclusion, we have explored the relationships between *Hepatozoon* infection and body condition in specimens of two Neotropical alligator species. We found that about 15% of *C. crocodilus* were infected with *Hepatoozon caimani*, whereas no *C. acutus* showed infection. We also have shown a greater infection in subadult individuals in *C. crocodilus*, which suggests that the infection could be provoked by the ingestion of paratenic hosts due the change in diet composition from the juvenile age. This change in diet with greater nutritional contribution may also explain the higher body condition of subadult individuals in both species of crocodiles. *Hepatozoon* infection varied significantly among study locations in *C. crocodilus*, highlighting the importance of handling and feeding conditions in captive crocodiles. Finally, *C. crocodilus* infected with *Hepatozoon* had higher body condition than the non-infected ones, and hence the link between body condition and *Hepatozoon* infection requires further investigations to be revealed. Despite our results showed limited negative impact of *Hepatozoon* infection on *C. crocodilus*, several studies have described anaemia, lethargy, decreased hematocrit, and continuous weight loss that may progress to death in crocodiles harbouring *Hepatozoon* (Viana et al. 2010a, Rojas et al. 2011, Campbell 2015). Therefore, longitudinal and experimental studies providing more information on the dynamics of *Hepatozoon* infection and its effects on fitness of crocodilians would be of great importance for the conservation–management actions of these endangered species.

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Data availability statement

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.pc866t1x5 (Marzal et al. 2024).

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