Blood parasites infecting the Hoatzin (*Opisthocomus hoazin*), a unique neotropical folivorous bird

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The Hoatzin (*Opisthocomus hoazin*) is the only extant member of the order Opisthocomiformes. This unique South American bird lives in the riparian lowland vegetation characteristic of the Amazon and Orinoco basins. Hoatzins nest in communal social units close to water bodies; they are strictly folivores being the only bird with pregastric fermentation in the crop. Because of the complex logistics involved in capturing this bird, there is a knowledge gap on its parasites. This study documents two distant lineages of haemosporidian parasites (*Plasmodium* spp.) in a juvenile and two adults sampled in the Cojedes state, Venezuela. Although negative by microscopy, the parasite identification was possible by using molecular methods. We estimated the phylogenetic relationships on the parasite cytochrome b (*cytb,* 480 bp) gene and the mitochondrial DNA. We found one of the parasites lineages in two individuals (nestling and adult), and the corresponding fragment of *cytb* was identical to a one found in Wood Stork (*Mycteria americana*) from Brazil. The other lineage, found in an adult, has an identity of 469 out of 478 bp (98%) with *Plasmodium* sp. GAL-2012 (isolate THAMB08) from Brazil. Although a morphological description of these parasites was not possible, this is the first molecular study focusing on Hoatzin haemosporidian parasites and the first documentation of *Plasmodium* infections in the Hoatzin from Venezuela. Furthermore, we reported microfilaria in two adults as well as hematological parameters for six individuals. Information on hematological parameters could contribute to establishing the necessary baseline to detect underlying conditions, such as infections, in this bird species.

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23   **Abstract**

24   The Hoatzin (*Opisthocomus hoazin*) is the only extant member of the order Opisthocomiformes.

25   This unique South American bird lives in the riparian lowland vegetation characteristic of the

26   Amazon and Orinoco basins. Hoatzins nest in communal social units close to water bodies; they

27   are strictly folivores being the only bird with pregastric fermentation in the crop. Because of the

28   complex logistics involved in capturing this bird, there is a knowledge gap on its parasites. This

29   study documents two distant lineages of haemosporidian parasites (*Plasmodium* spp.) in a juvenile

30   and two adults sampled in the Cojedes state, Venezuela. Although negative by microscopy, the

31   parasite identification was possible by using molecular methods. We estimated the phylogenetic

32   relationships on the parasite cytochrome b (*cytb,* 480 bp) gene and the mitochondrial DNA. We

33   found one of the parasites lineages in two individuals (nestling and adult), and the corresponding

34   fragment of *cytb* was identical to a one found in Wood Stork (*Mycteria americana*) from Brazil.

35   The other lineage, found in an adult, has an identity of 469 out of 478 bp (98%) with *Plasmodium*

36   sp. GAL-2012 (isolate THAMB08) from Brazil. Although a morphological description of these

37   parasites was not possible, this is the first molecular study focusing on Hoatzin haemosporidian

38   parasites and the first documentation of *Plasmodium* infections in the Hoatzin from Venezuela.

39   Furthermore, we reported microfilaria in two adults as well as hematological parameters for six

40   individuals. Information on hematological parameters could contribute to establishing the

41   necessary baseline to detect underlying conditions, such as infections, in this bird species.

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INTRODUCTION

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47   The Hoatzin (*Opisthocomus hoazin*), the only extant species of the Order Opisthocomiformes, is

48   a unique bird native to the Orinoco and the Amazon basins in South America that includes Bolivia,

49   Colombia, Ecuador, Peru, Venezuela, Brazil, and the lowlands of the Guianas. Little is known

50   about its ecology and evolution. The lack of information is due in part to difficulties accessing its

51   habitat, as well as the behavior and size of these birds that makes them hard to capture. In

52   Venezuela, this species is distributed along rivers of the central savannas to the eastern Orinoco

53   River. They live in colonies in riverine and swamp forest, vegetation at edges of ponds, oxbow

54   lakes, and other freshwater wetlands. It is folivore bird that feeds on riverine tree species, and nests

55   in communal social units building their nest close to water bodies (Thomas, 1996). Its phylogenetic

56   relationship remains an enigma (Jarvis et al., 2014; Claramunt & Cracraft, 2015; Prum et al., 2015).

57   However, given that is the only bird with pregastric fermentation in the crop like that in ruminants

58   (Grajal et al., 1989), most of the knowledge about this species is on the crop microorganism's

59   community structure and ecology (e.g., Godoy-Vitorino et al., 2010, 2012; Bardele et al., 2017).

60   Indeed, there are only a few studies on ectoparasites (Hernandes & Mironov, 2015; Bauchan et al.,

61   2017) and hemoparasite infections in the Hoatzin (Renjifo et al., 1952; Gabaldon, 1998). Only

62   filarial parasite infections have been reported in the Hoatzin from Colombia (Renjifo et al., 1952),

63   and no haemosporidian parasites had been found before in this species using blood films (Renjifo

64   et al., 1952; Gabaldon, 1998).

65   In this study, we report two distant molecular lineages of haemosporidian parasites of the genus

66   *Plasmodium* (Family Plasmodiidae, Order Haemosporida, Phylum Apicomplexa) found in the

67   Hoatzin from the Cojedes River, a tributary of the Orinoco River in central Venezuela that is part

68   of the Orinoquia. Plasmodiidae is a diverse group of vector-borne haemoparasites found in many

69   terrestrial vertebrate hosts (Garnham, 1966; Valkiūnas, 2005; Telford, 2009), including the species

70   of human (Cavalier-Smith, 2014) and avian malaria (e.g. *Plasmodium relictum*; Bensch et al. 2009;

71   Atkinson & Samuel, 2010). This is the first evidence of *Plasmodium* infections in the Hoatzin in

72   South America; additionally, we provided hematological parameters to generate information that

73   may help to assess the health status of this bird species.

74

75   **MATERIAL AND METHODS**

76   **Study area and samples**

77   We caught eight individuals in *El Baúl* massif along the Cojedes River near the town of *El Baúl*

78   located at the southwestern of the Cojedes state in the northcentral Venezuela. This is considered

79   part of the Orinoquia region at the north of the Guyana Shield. *El Baúl* massif is about 720 km2;

80   it is relatively isolated and mountainous with steep topography following a northwest-southeast

81   trend (Viscarret et al., 2009). The vegetation is typical of the Orinoquia sedimentary and alluvial

82   plains (e.g., vegetation of savannas, gallery forests, palm groves, and semi-deciduous forests). In

83   the context of this study, the birds were captured in the gallery forest, which is moderately

84   intervened and subject to seasonal flooding from the Cojedes River as well as forest fires

85   (González-Fernández et al., 2007).

86   We trapped two individuals (one adult and one juvenile) in October 2010, and 6 individuals

87   (two adult and 4 nestlings) in August 2015 in their nest during nighttime using butterfly nets and

88   transported to the field laboratory. From each bird, we obtained blood samples by brachial vein

89   puncture, and then, immediately we prepared three to five thin smears. We preserved the rest of

90   the sample in protein saver cards (Whatman 903, Whatman™, Cardiff, UK) for molecular analysis.

91   We collected the specimens under permit number 0950 issued by the Venezuelan government

92   (*Oficina Nacional de Diveridad Biologica, Ministerio del Poder Popular para Ecosocialismo,*

93   *Hábitat y Vivienda*). All the animal protocols were approved by the ethics committees of *Instituto*

94   *Venezolano de Investigaciones Científicas* (IVIC, Venezuela) under the number COBIANIM N°

95   Dir-0885/1517/2014.

96   **Examination of blood films**

97   Smears were air-dried immediately after preparation, fixed in absolute methanol for 5 min, and

98   then stained with Giemsa (pH 7.2) for 45 min. Using a Leica DM750e microscope (Leica

99   Microsystems, Heerbrugg, Switzerland), we first examined blood slides at x400 for 10 min and

100   then at x1000 for 20 min. For the capture of digital images, we scanned entirety those slides with

101   hemoparasites using a Leica EC3 digital camera and processed with the LAS EZ (Leica

102   Microsystems Suiza Limited 2012). Then, we estimated the intensity of infection as No. of

103   parasites/10000 erythrocytes from erythrocyte counts with an increase of x1000, focusing on areas

104   where blood cells formed a monolayer (Muñoz et al., 1999). In addition, using ImageJ software

105   (Schneider et al., 2012), we performed morphometric analyses of the erythrocytes. We measured

106   the maximum cell width and length, as well as nuclear width and length for 30 erythrocytes per

107   slide and per individual, following Hartman and Lessler (1963). To estimate the percentage of each

108   type of white blood cell present in blood, we also measured a differential white blood cell (WBC)

109   count per 100 cells using the blood samples collected in 2015 following the protocol by Clark

110   (2009). We obtained all these measures for only those individuals caught in 2015 (*N*=6) because

111   of the better quality of their blood films.

112   **Molecular diagnostic of haemosporidian parasites**

113   We extracted genomic DNA from whole blood using QIAamp® DNA Micro Kit (Qiagen GmbH,

114   Hilden, Germany). We screened each sample for haemosporidian parasites by using a nested

115   polymerase chain reaction (PCR) protocol that targets the parasite mitochondrial cytochrome b

116   (*cytb*, 1,131 bp) gene using the primers described in Pacheco et al., (2011, 2018). The *cytb* external

117   primers were forward AE298 5′-TGT AAT GCC TAG ACG TAT TCC 3′ and reverse AE299 5′-

118   GT CAA WCA AAC ATG AAT ATA GAC 3′, and the internal primers were forward AE064 5′-

119   T CTA TTA ATT TAG YWA AAG CAC 3′ and reverse AE066 5′-G CTT GGG AGC TGT AAT

120   CAT AAT 3′. The primary PCR amplifications were carried out in 50 µl volume reaction using 5-

121   8µl of total genomic DNA, 2.5 mM $MgCl_2$, 1X PCR buffer, 1.25 mM of each deoxynucleoside

122   triphosphate, 0.4 mM of each primer, and 0.03 U/µl AmpliTaq polymerase (Applied Biosystems,

123   Roche-USA). The primary PCR conditions were: A partial denaturation at 94 °C for 4 min and 36

124   cycles with 1 min at 94 °C, 1 min at 53 °C and 2 min extension at 72 °C, and we added a final

125   extension of 10 min at 72 °C in the last cycle. Then, the nested PCRs were also made in 50 µl

126   volume reaction using only 1 µl of the primary PCRs, 2.5 mM $MgCl_2$, 1 × PCR buffer, 1.25 mM

127   of each deoxynucleoside triphosphate, 0.4 mM of each primer, and 0.03 U/µl AmpliTaq

128   polymerase. The nested PCR conditions were: A partial denaturation at 94 °C for 4 min and 25

129   cycles with 1 min at 94 °C, 1 min at 56 °C and 2 min extension at 72 °C, and we also added a final

130   extension of 10 min at 72 °C in the last cycle. Both strands for all the *cytb* fragments were directly

131   sequenced using an Applied Biosystems 3730 capillary sequencer. We identified all the *cytb*

132   fragments obtained here as *Plasmodium* using BLAST (Altschul et al., 1997).

133   For those samples that were positive using the *cytb* PCR protocol, we amplified between 5515

134   to 5838 bp of the parasite mitochondrial genomes (mtDNA) using a nested PCR with Takara LA

135   Taq™ Polymerase (TaKaRa Takara Mirus Bio) following manufacturers' directions. This

136   fragment of the mtDNA included the three nonprotein coding regions between the ORFs

137   (fragmented SSU rRNA and LSU rRNA) and the three protein-coding genes (*Cox3, Cox1* and

138   *Cytb*) so only three tRNAs (7, 11, and 14) and two fragments of small subunit ribosomal RNAs (5

139   and 7) are missing. Oligos forward AE170 5′ GAGGATTCTCTCCACACTT

140   CAATTCGTACTTC 3′ and reverse AE171 5′ CAGGAAAATWA

141   TAGACCGAACCTTGGACTC 3′ were used for the primary PCR and internal oligos forward

142   AE176 5′ TTTCATCCTTAAATCTCGTAAC 3′ and AE136 reverse 5′ GACCGAA

143   CCTTGGACTCTT 3′ for the inner PCR. The PCR conditions were a partial denaturation at 94 °C

144   for 1 min and 30 cycles with 30 sec at 94 °C and 7 min at 68 °C and a final extension of 10 min at

145   72 °C. Then, we excised two independent PCR products (50ul) from the gel (bands of

146   approximately 2 kbp) and purified using QIAquick® Gel extraction kit (Qiagen, GmbH, Hilden,

147   Germany). We cloned at least two independent PCR products using pGEM®-T Easy Vector

148   Systems (Promega, USA), and we sequenced four clones from each individual. We sequenced both

149   strands for PCR products and clones using an Applied Biosystems 3730 capillary sequencer. Given

150   that the *cytb* partial sequences and the *cytb* gene from the mtDNA genome were 100% identical

151   for each sample, we only deposited the mtDNA genome sequences in GenBank under the

152   accession numbers KY653749 to KY653751.

153   **Phylogenetic analysis of the *cytb* fragment and mtDNA genome**

154   We performed two different nucleotide alignments by using ClustalX v2.0.12 and Muscle as

155   implemented in SeaView v4.3.5 (Gouy et al., 2010) with manual editing. The first alignment was

156   constructed with 45 *cytb* partial sequences (480 bp) belonging to three genera (*Leucocytozoon*,

157   *Haemoproteus*, and *Plasmodium*). This alignment included the sequences obtained in this study as

158   well as sequences from well-known parasite species based on morphology (Valkiūnas and Iezhova

159   2018) that were available in GenBank (Benson et al., 2012) and MalAvi (Bensch et al., 2009)

160   databases at the time of this study. Sequences that showed a similarity > 95% using BLAST

161   (Altschul et al., 1997) were also included even when they are not clearly linked to the described

162   species. The second alignment (5286 bp excluding gaps) was done using 31 mtDNA genome

163   sequences belonging also to the three genera, including the sequences obtained in this study and

164   sequences from well-known parasite species (using morphology) available in GenBank.

165   Subsequently, the alignment was divided into six partitions corresponding to the three non-protein

166   coding regions between the ORFs (fragmented SSU rRNA and LSU rRNA) and the three protein-

167   coding genes, keeping their order in the mtDNA genome (nonprotein coding, *cox3*, nonprotein

168   coding, *cox1*, *cytb*, nonprotein coding).

169   Then, we inferred phylogenetic hypotheses based on the first (partial *cytb* gene) and second

170   (mtDNA genome) alignments using the Bayesian methods implemented in MrBayes v3.2.6 with

171   the default priors (Ronquist & Huelsenbeck, 2003). To estimate the phylogenetic hypothesis that

172   best fit the data, we used the general time reversible model with gamma-distributed substitution

173   rates and a proportion of invariant sites (GTR + Γ +I) on the *cytb* alignment and for each partition

174   in the mtDNA genome alignment. This model was the one with the lowest Bayesian Information

175   Criterion (BIC) scores for both alignments and each partition as estimated by MEGA v7.0.14

176   (Kumar et al., 2016). We inferred Bayesian support for the nodes in MrBayes by sampling every

177   1000 generations from two independent chains lasting $4X10^6$ Markov Chain Monte Carlo

178   (MCMC) steps. The chains were assumed to have converged once the value of the potential scale

179   reduction factor (PSRF) was between 1.00 and 1.02 and the average SD of the posterior probability

180   was <0.01 (Ronquist and Huelsenbeck 2003). Then, we discarded 25% of the sample once

181   convergence was reached as a "burn-in". For both phylogenies, we used *Leucocytozoon* species as

182   out-group. Genbank accession numbers for all sequences used in the analyses are given in the

183   phylogenetic trees.

184

185   **RESULTS**

186   In this study, one sample was positive for haemosporidian parasites by microscopy (adult caught

187   in 2015). The individual had only *Plasmodium* sp. trophozoites in its blood films (Fig. 1), so a

188   morphological description of this parasite was not possible. Indeed, the parasitemia was very low

189   (<0.01), consistent with a subpatent infection. In addition, the two adults caught in 2015 were

190   infected with microfilariae, so one of the adults has a coinfection of filarial parasites and

191   *Plasmodium* sp. (Fig. 1). Unfortunately, the identification of nematode microfilariae at species

192   level is difficult given their high degree of morphological and morphometric similarities

193   (McKeand, 1998). None of the nestlings were infected with hemoparasites. Morphometry of the

194   uninfected erythrocytes is shown in Table 1. We compared these measurements with those from

195   the erythrocytes of other avian species with similar body size. In addition, we provided differential

196   white blood cell (WBC) count profiles in Table 2.

197   The molecular diagnostic detected that the two individuals caught in 2010 (one adult and one

198   juvenile) and one of the adults caught in 2015 (the one positive by microscopy) were positive by

199   nested PCR (3/8, 37.5%). In order to characterize these *Plasmodium* species, for those individuals

200   (*N*=3) that were positive by nested PCR, we obtained the parasite mtDNA genome sequences. We

201   further examined these sequences by using phylogenetic analyses (Fig. 2) yielding two estimated

202   gene trees, one just with partial *cytb* sequences commonly used to identify haemosporidia parasites

203   (Bensch et al., 2009; Pacheco et al., 2018) and the other with mtDNA genome (Pacheco et al.,

204   2018). These phylogenies have similar topologies. We found two lineages of *Plasmodium*

205   (identified as 1 and 2 in Fig. 2) in three individuals of Hoatzin. The individuals caught in 2010

206   (one adult and one juvenile) were infected with the same *Plasmodium* lineage (KY653750-

207   KY653751), and their mtDNA genome sequences were 100% identical.

208

209   **DISCUSSION**

210   This is the first report of *Plasmodium* species in this bird and the first record of microfilarias in

211   Hoatzins from Venezuela. We have no sign of pathogenesis associated with these infections.

212   However, as numbers of heterophils and lymphocytes could be affected by stress such as a parasitic

213   infection, the ratio of one to the other (H:L) is commonly used as a stress indicator (Gross & Siegel,

214   1983; Maxwell, 1993). Thus, we reported the H:L ratio since it is expected to increase in response

215   to stressors such as infectious diseases (Davis et al., 2008), especially in birds with high

216   parasitemia (Granthon & Williams, 2017). Here, the bird infected with filarial parasites and

217   *Plasmodium* sp. had an increase in the number of lymphocytes and a decrease in the number of

218   heterophils in comparation with the haemosporidian negative birds (infected only with microfilaria

219   and uninfected individuals), and so a lower H:L ratio (1.5 vs. 2.4, Table 2). The opposite occurred

220   in the individual infected only with filarial parasites (H:L=3.9 vs. 2.4, Table 2). WBC counts are

221   difficult to interpret within and between species (e.g., Ricklefs & Sheldon, 2007) so these results

222   should be taken with caution especially considering that these are a few individuals from a small

223   sample. Considering the paucity of data from this species, we provided the WBC count measures

224   for comparison in future studies. Although it has been hypothesized an association between

225   erythrocyte size and the species body size as result of differences in their metabolic rates (Hartman

226   & Lessler, 1963), we found that erythrocyte size from Hoatzins was comparable to those reported

227   from putative sister taxa like Gruiiformes (Jarvis et al., 2014; Claramunt & Cracraft, 2015),

228   Charadriiformes (Claramunt & Cracraft, 2015), and Accipitriformes (Prum et al., 2015) (Table 1).

229 Furthermore, differential white blood cell (WBC) count profiles (Table 2) from non-infected

230 individuals showed marked similitude with those reported in nonpasserine birds such as cranes,

231 raptors, and vultures (Davis, 2009).

232   Wherever there are subpatent infections, it is difficult to determine the prevalence of

233 haemosporidian parasites since those infections are usually submicroscopic. In such cases,

234 parasites can only be detected by polymerase chain reaction using genes with high copy number

235 like *cytb* (Pacheco et al., 2018). The molecular diagnostic detected two lineages of *Plasmodium*

236 genus (Fig. 2) in these three individuals of Hoatzin. Interesting, the lineage found in the individuals

237 caught in 2010 (one adult and one juvenile) has been only reported so far in Brazil (Villar et al.,

238 2013; Ferreira et al., 2017; Tostes et al., 2017) suggesting that it is distributed solely in South

239 America. In particular, its partial *cytb* sequence match 100% with a parasite lineage reported in

240 several bird species: MYCAME02 isolated from Wood Storks nestlings found in the northern

241 region of Brazil (*Mycteria Americana*, Pelecaniformes)(Villar et al., 2013), H2 isolate from

242 Streaked Flycatcher found in Southeastern Brazil (*Myiodynastes maculatus*,

243 Passeriformes)(Ferreira et al., 2017), and lineages reported from birds belonging to the orders

244 Strigiformes, Accipitriformes, and Falconiformes kept in captivity in Southeaster Brazil (Tostes

245 et al., 2017). In the phylogenetic analysis, this lineage appears as the sister taxon of *Plasmodium*

246 *nucleophilum*, parasite isolated from an Egyptian Goose in São Paulo Zoo, Brazil (Chagas et al.,

247 2013). Both parasites, the lineage reported here and *P. nucleophilum*, are part of a monophyletic

248 group that includes *Plasmodium ashfordi* and *Plasmodium delichoni*. This monophyletic group is

249 at the base of the mitochondrial phylogeny of the known Avian *Plasmodium* parasites. Given the

250 lack of gametocyte data, it is possible that the *Plasmodium* infections in these two Hoatzin

251 individuals were abortive, but the fact that the two individuals (caught together) were infected with

252   the same parasite suggests that its transmission is occurring, and the parasite lineage is circulating

253   in Cojedes State, Venezuela. Tostes et al., (2017) re-described the parasite linked to this *cytb*

254   lineage as *Plasmodium* (*Novyella*) *paranucloephilum*, a species originally described by Manwell

255   & Sessler (1971) in a South American tanager of uncertain species likely from northern Brazil.

256   However, given the absence of morphological data in this study, and that there is no *cytb* neither

257   other mtDNA genome sequences belonging to the original parasite description, we consider that it

258   is premature to identify the lineage found in this study as *P. paranucloephilum*. The identity of

259   this lineage could be established when more samples from birds with high parasitemia of this

260   haemosporidian parasite become available.

261   Regarding the second lineage found infecting hoatzin (KY653749, Fig. 2), it appears as the

262   common ancestor of a clade that includes *Plasmodium tejerai*, *Plasmodium matutinum* and

263   *Plasmodium lutzi*. This clade forms a monophyletic group with *P. elongatum* (Fig. 2); one of the

264   most pathogenic and generalist avian malaria parasite worldwide (Palinauskas et al., 2016). Given

265   that we did not find any sequence with 100% similarity with our lineage in the available databases,

266   this result indicates that likely a new parasite is circulating in the area. Considering the difficulty

267   of catching this bird species, it is worth noticing that even with this small sample size (only eight

268   individuals including four nestlings) we found two *Plasmodium* lineages infecting the Hoatzins.

269   Riparian zones from the Orinoco and Amazon basins are considered important in terms of their

270   biodiversity since they result from variable flood regimes, geographically unique channel

271   processes, altitudinal climate shifts, and upland influences on fluvial corridors. Furthermore, these

272   areas are treated as critical habitat to several endangered bird species, refuges to the fauna

273   inhabiting adjacent areas and, in some cases, hotspots and corridors for bird migration and

274   dispersal (Naiman & Decamps, 1997; Franchin et al., 2009). In the Amazonia, the ability of avian

275   malaria parasites to disperse geographically and shift among avian hosts have been played a role

276   in their radiation and have shaped their current distributions and diversity (Sebaio et al., 2012;

277   Fecchio et al. 2018a; 2018b). Thus, the fact that one of the lineages reported in the Hoatzin has

278   been found also in species in Brazil is consistent with this notion of broad geographic distribution

279   and multiple hosts. This finding also indicate that the parasite communities in the Orinoquia and

280   the Amazon basin share species so, at this point, we can only speculate that similar processes may

281   shape the parasite communities in both areas.

282   Riparian ecosystems in the Orinoquia are in a state of dynamic flux due to human interventions,

283   seasonal flooding, and fires. In addition, these areas are suitable for insects that could act as

284   vectors. Considering all these factors, the Orinoquia should be given special priority for future

285   research in order to document its parasite-avian host ecology and biodiversity.

286

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290   laboratory at the School of Life Sciences (Arizona State University) for their technical support.

291

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433

Comparison of erythrocyte measurements from different bird orders including the values for Hoatzin.

The values for Hoatzin (*O. hoazin*) are from six individuals caught in August 2015 at the Cojedes River, Venezuela.*Erythrocyte measurements are from Hartman and Lessler (1963) and &from Tostes et al. (2017).

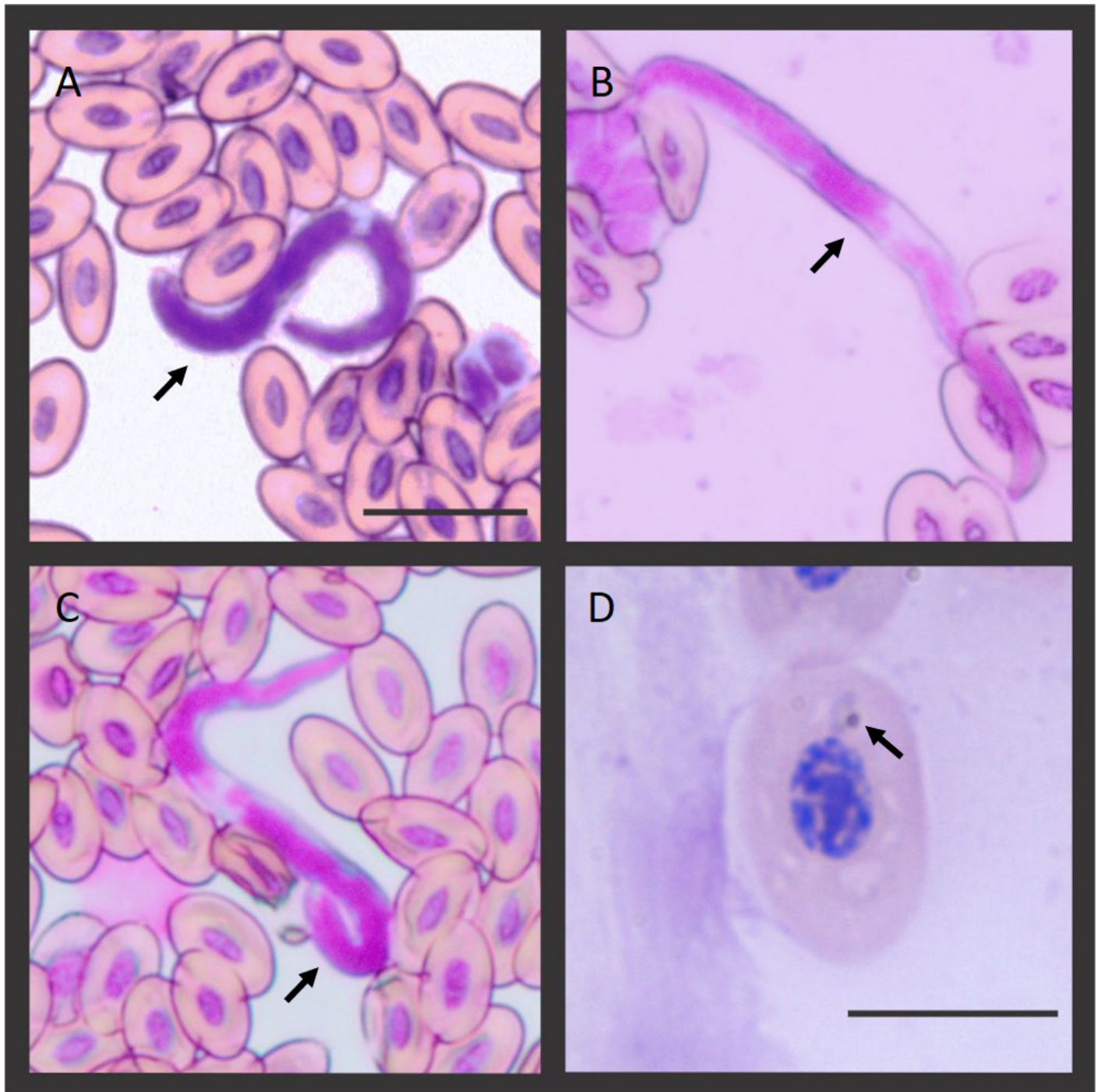
	Cytosome			Nucleus		
	Length (µm)	Width (µm)	Ratio (L/W)	Length (µm)	Width (µm)	Ratio (L/W)
Opisthocomiformes						
O. hoazin	15.3 ± 0.85	8.7 ± 0.71	1.76	6.2 ± 0.61	3.4 ± 0.47	1.82
Gruiformes*						
Rallus elegans	14.5 ± 0.32	7.7 ± 0.17	1.89	5.7 ± 0.13	2.9 ± 0.12	1.97
Aramides cajanea	12.9 ± 0.78	7.2 ± 0.50	1.79	5.5 ± 0.40	3.3 ± 0.37	1.67
Fulica americana	11.4 ± 0.26	7.5 ± 0.22	1.51	4.2 ± 0.06	2.3 ± 0.07	1.83
Charadriiformes*						
Jacana spinosa	13.7 ± 0.79	7.5 ± 0.52	1.83	5.9 ± 0.64	3.7 ± 0.33	1.59
Charadrius wilsonia	12.8 ± 0.27	7.3 ± 0.13	1.76	5.8 ± 0.11	2.4 ± 0.05	2.41
Himantopus mexicanus	12.8 ± 0.16	6.9 ± 0.18	1.85	5.8 ± 0.20	2.5 ± 0.99	2.32
Accipitriformes*						
Accipiter cooperii	14.3 ± 0.27	8.1 ± 0.13	1.77	6.2 ± 0.14	2.4 ± 0.11	2.58
Buteo platypterus	13.4 ± 0.51	7.6 ± 0.34	1.77	6.2 ± 0.31	3.0 ± 0.32	2.07
Rupornis magnirostris[&]	13.1 ± 0.72	7.4 ± 0.36	1.78	6.83 ± 0.38	2.84 ± 0.18	2.4

Differential white blood cell (WBC) counts and H:L ratio in the Hoatzins (*N*=6) caught in August 2015 at the Cojedes River, Venezuela.

	Uninfected nestlings	Infected adults	
	(*N*=4)	microfilaria (*N*=1)	microfilaria/*Plasmodium* (*N*=1)
Heterophils	64.25 ± 2.75	67	53
Lymphocytes	27 ± 1.83	17	35
Monocytes	5 ± 0.82	8	5
Eosinophils	3.25 ± 0.5	7	5
Basophils	0.5 ± 0.58	1	1
H:L	2.4	3.9	1.5

Figure 1

Microfilaria and *Plasmodium* sp. blood stage infecting the Hoatzins (*O. hoazin*) caught in August 2015 at the Cojedes River, Venezuela.

(A) Hoatzin individual infected only with filarial parasites. (B*), (C*), and (D&) Hoatzin individual infected with filarial parasites and *Plasmodium* sp. *Microfilarial stages of the filarial parasite (Scale bar = 20 µm) and &*Plasmodium* sp. trophozoite (Scale bar = 10 µm). We indicated the hemoparasite stages by the black arrows.

A Bayesian phylogenetic hypothesis of *Plasmodium* parasites infecting the Hoatzins (*O. hoazin*) caught at the Cojedes River, Venezuela.

We constructed phylogenetic trees based on parasites (A) partial sequences of the *cytb* gene (45 sequences and 480 bp excluding gaps) and mtDNA genomes (31 sequences and 5286 bp excluding gaps). The values above branches are posterior probabilities (see "Material and methods"). *Leucocytozoon* genus (outgroup) is indicated in grey. We provided in parentheses both lineages (as deposited in the MalAvi database) and their Genbank accession numbers for all the sequences used in the analyses.