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Eurasian Magpies (*Pica pica*) and Carrion Crows (*Corvus corone*) as a reservoir of avian Plasmodium that infect captive African Black-footed Penguins (*Spheniscus demersus*) at La Palmyre Zoo: a comparative morphological and molecular study

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1 **Eurasian Magpies (*Pica pica*) and Carrion Crows (*Corvus***
2 ***corone*) as a reservoir of avian *Plasmodium* that infect captive**
3 **African Black-footed Penguins (*Spheniscus demersus*) at La**
4 **Palmyre Zoo: a comparative morphological and molecular**
5 **study.**

6

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9

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19

20

21

22 **Abstract**

23 A morphological and molecular epidemiological investigation was conducted on a captive
24 African Black-footed Penguin (*Spheniscus demersus*) colony with a history of *Plasmodium*
25 infections at La Palmyre Zoo (France). Although *Plasmodium* parasites were detected in
26 blood smears and tissues collected from the penguins, various blood parasites, including
27 several *Plasmodium* spp. were recorded in blood smears from wild Eurasian Magpies
28 (*Pica pica*) and Carrion Crows (*Corvus corone*) sampled at the same time in the study area.
29 Nonetheless, nested polymerase chain reaction (nt-PCR) enabled detection of *Plasmodium*
30 DNA in 28/44 (64%) penguins, 15/26 (58%) magpies, and 4/9 (44%) crows. Sequencing
31 and phylogenetic analyses indicated that the parasite DNAs amplified from the penguins,
32 magpies and crows were similar.

33

34

35 **Key words**

36 *Corvus corone*, malaria, *Pica pica*, *Plasmodium*, polymerase chain reaction, *Spheniscus*
37 *demersus*.

38

39

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42 **Introduction**

43

44 *Plasmodium* species in penguins were first reported in 1927 by SCOTT, who observed
45 several stages of *Plasmodium praecox* (*P. relictum*) in the peripheral blood of a King
46 Penguin (*Aptenodytes patagonicus*) at the Zoological Society's Gardens in London
47 (SCOTT 1927). The presence of *Plasmodium* infections in Sphenisciforms have since
48 been frequently recorded in rehabilitation centers (BROSSY 1992, PARSONS
49 & UNDERHILL 2005) and in wild (GRACZYK *et al.* 1995) and captive (RODHAIN
50 1937, 1938, 1939, FLEISCHMAN *et al.* 1968, FIX *et al.* 1988, CRANFIELD *et al.* 1990,
51 GRACZYK *et al.* 1994, McCONKEY *et al.*, GRACZYK *et al.* 1995, PETIT 1998,
52 LECLERC 2008) penguin colonies, where they were often associated with high
53 morbidity and mortality (FIX *et al.* 1988, CRANFIELD *et al.* 1990, PETIT 1998,
54 LECLERC 2008). In particular, the species *Spheniscus demersus* has often been reported
55 as a host of the parasite (BENNETT *et al.* 1992, BROSSY 1993, BROSSY *et al.* 1999,
56 DUIGNAN 2001, FANTHAM & PORTER 1944, FIX *et al.* 1988, FLEISHMAN *et al.*
57 1968a, GRACZYK *et al.* 1995a, JONES & SHELLAM 1999a, 1999b, LOMBARD *et al.*
58 1999, MILLER *et al.* 2001, PARSONS & UNDERHILL 2005, CRANFIELD *et al.* 1990,
59 GRACZYK & CRANFIELD 1996, GRACZYK *et al.* 1994a, 1994b, 1994c, 1994d,
60 GRINER 1974, 1983, McCONKEY *et al.* 1996, PETIT 1998, RODHAIN 1938, 1939,
61 RODHAIN & ANDRIANNE 1952, STOSKOPF & BEALL 1980, STOSKOPF & BEIER
62 1979, BROSSY 1992, GRIM *et al.* 2003, LOMBARD *et al.* 1999).

63 La Palmyre Zoo has hosted several penguin species since 1985 and ten cases of lethal
64 *Plasmodium* infections have been confirmed in *Spheniscus demersus* and *Spheniscus*

65 *magellanicus*. Seventeen other cases had been suspected but confirmation was not
66 provided (LECLERC 2008).

67 Chemical prophylaxis was initiated in the late 1990's to diminish the losses due to
68 *Plasmodium* infections in the penguin colony. Since 2000 pyrimethamine (Malocide,
69 Sanofi-aventis France, 75017, Paris, France, 12,5 mg *per os* per penguin) has been given
70 twice a week from April to November (LECLERC 2008). It is not sure that every
71 penguin of the colony properly received its dose at each distribution since fishes
72 containing pyrimethamine were less regularly eaten, particularly by older penguins.
73 However, no further penguin deaths were subsequently attributed to *Plasmodium*
74 infections at La Palmyre. To date, the avian reservoir of the infection had not been
75 identified and this would be desirable because a good understanding of the infection
76 epidemiology would enhance the prophylactic measures.

77 The aim of this study was to *i)* to establish whether *Spheniscus demersus* individuals
78 under chemical prophylaxis at La Palmyre Zoo harbored *Plasmodium* infections ; *ii)* to
79 identify potential reservoirs of the infection among local wild birds. We focused on two
80 birds species classified as pest that are present on site: the Eurasian Magpie (*Pica pica*)
81 and the Carrion Crow (*Corvus corone*) because a high prevalence of *Plasmodium* had
82 been previously reported in their populations elsewhere in France (CHAVATTE *et al.*
83 2007). To this end, morphological and molecular investigations were carried out and
84 comparative analyses were carried out on the *Plasmodium* parasites observed in
85 *Spheniscus demersus*, *Pica pica* and *Corvus corone*.

86

87 **Material studied, area descriptions, methods, techniques**

88

89 The study was conducted at La Palmyre Zoo (45°41'N, 01°09' to 01°10'W) between
90 November 2006 and August 2007.

91

92 *Penguins*

93 44 African Black-footed Penguins (*i.e.* the whole colony) were studied. They were
94 undergoing pyrimethamine prophylaxis on and after April 1st 2007. Blood samples were
95 collected on three occasions (November 16th 2006, May 4th 2007 and July 25th 2007) and
96 on several other occasions on sick animals. 0.2 to 1 mL of blood was drawn from the
97 right jugular vein, the coccygian plexus or a digital vein using a 1 mL syringe containing
98 0.01 mL ethylenediaminetetraacetic acid (EDTA). Blood smears were also prepared in
99 duplicate on the spot by spreading one drop of blood onto a glass slide, which was
100 immediately air-dried. Tissue samples were collected from penguins that died during the
101 period of the study. These consisted of heart, lung, liver, spleen and kidney samples that
102 were immediately immersed in Carnoy's fluid (60% absolute ethanol, 30% chloroform,
103 10% acetic acid). A total of 186 blood smears (93 duplicates), 51 blood samples and 6
104 sets of tissue samples were analyzed.

105

106 *Eurasian Magpies & Carrion Crows*

107 Eurasian Magpies and Carrions Crows are classified as pests in the area of study.
108 Therefore, in accordance with the French legislation an official trapping permit was
109 delivered, and appellant traps were used for capture. 26 Eurasian Magpies and 9 Carrion

110 Crows were trapped. They were identified, and placed in an aviary. Three to five days
111 later, duplicate blood smears were prepared from a blood drop pressed out of a foot claw
112 after a small incision was made. Blood was also collected from the brachial vein or the
113 occipital plexus in an EDTA-containing syringes. Magpies and crows were then
114 euthanized by an occipital injection of embutramide, mebezonium and tetracaine (T61,
115 MSD Santé Animale, 49071 Beaucouze Cedex, France) and tissues (heart, lung, liver,
116 spleen, kidney) were collected in Carnoy's fluid. A total 94 blood smears (47 duplicates),
117 34 blood samples and 36 sets of tissue samples were analyzed.

118

119 *Morphological study*

120 Blood smears were sent to the Muséum National d'Histoire Naturelle for morphological
121 examination within 48h of their preparation. On reception, they were immediately fixed
122 in 100% methanol and stained for 1 hour with 10% Giemsa in a phosphate buffer at pH
123 7.2. A cover-slip was mounted on each smear with Eukitt® medium prior reading. The
124 slides were observed using an optical microscope under x400 magnification and x1000
125 immersion magnification. Parasitaemias were estimated in a semi-quantitative way.

126 Tissue samples were embedded in paraffin wax and cut at 500 nm. Paraffin wax was
127 cleared by acetone-toluene baths and sections deposited on slides were stained for 40
128 minutes to one hour using the Giemsa-Collophonium differentiation method (BRAY &
129 GARNHAM 1962). Smears were read using an optical microscope under x400
130 magnification and x1000 immersion magnification.

131

132 *Molecular study*

133 Blood samples were frozen and stored at -18 °C until the end of the sampling period.
134 They were then sent to the Muséum National d'Histoire Naturelle for molecular analysis.
135 DNA was classically extracted using the QIAamp® DNA Mini Kit (QIAgen, Germany).
136 Amplification of a fragment of the 18S ssrRNA gene was performed by a nested
137 polymerase chain reaction (nt-PCR) (SNOUNOU *et al.* 1993). Basically, it is a two step
138 PCR where 1 µL of the PCR products obtained at the initial step with the primers pair
139 rPLU1 (TCAAAGATTAAGCCATGCAAGTGA) / rPLU5
140 (CCTGTTGTTGCCTTAAACTTC) were used as template for the second nested
141 reactions performed with the primers pair rPLU3
142 (TTTTTATAAGGATAACTACGGAAAAGCTGT) / rPLU4
143 (TACCCGTCATAGCCATGTTAGGCCAATACC). If this reaction displayed positive
144 results several other nested reactions could be performed with several other sets of
145 primers so as to amplify larger or specific fragments of the gene. The initial reaction was
146 carried out in a total volume of 40 µL containing 1X PCR Buffer, 2.5mM of MgCl₂, 0.8
147 U of AmpliTaq® DNA Polymerase (Invitrogen™), 125 µM of each dNTP, 0.25 µM of
148 each primers and 1 µL of DNA template. The second reactions were carried out in a total
149 volume of 20 µL containing 1X PCR Buffer, 2m M of MgCl₂, 0.8 U of AmpliTaq® DNA
150 Polymerase (Invitrogen™), 200 µM of each dNTP, 0.3 µM of each primers and 1 µL of
151 PCR product as template. For the initial reaction, following a denaturation at 95°C for 5
152 min, the cycling conditions were 58 °C for 2 min, 72 °C for 2 min and 94 °C for 1 min
153 (25 cycles) and a final extension at 72 °C for 5 min; for the nested reaction the cycling
154 conditions were similar except that 30 cycles were carried out and the final extension at
155 72°C lasted 15 min.

156

157 Electrophoresis on a 1.5% agarose gel was carried out to separate DNA fragments and
158 compare their size to that of standard DNA markers. DNA was then purified using the
159 QIAquick® Gel Extraction Kit (QIAGEN, Germany). Amplified and purified fragments
160 were cloned using the TOPO TA Cloning® kit for sequencing (Invitrogen, USA),
161 Bacterial colonies were screened for the presence of an insert using universal primers
162 M13 oligonucleotides and positive colonies were cultured overnight in liquid medium
163 containing ampicillin. Plasmid purification was made with the QIAprep® Spin Miniprep
164 Kit (QIAGEN, Germany). The cloned DNA fragments were sequenced at the Sequencing
165 Platform of the Institut Cochin, Paris, France. Each sequence was obtained from
166 fragments obtained in duplicate from independent amplification reactions. Corrections by
167 cross-checking and alignment were made using BioEDIT® (Ibis Biosciences), ApE
168 (Wayne Davis) and Seaview 4 (GOUY *et al.* 2010). DNA sequences were compared to
169 published sequences of the *Plasmodium* 18S ssrRNA gene by Basic Local Alignment
170 Search Tool (BLAST).

171 In a previous study of the diversity of *Plasmodium* parasites of the Eurasian Magpie
172 (CHAVATTE *et al.* 2007), 5 haplotypes of *Plasmodium* arbitrary named (A, B, C, D and
173 E) were isolated (GenBank accession numbers KF061241 to KF061245). For each of
174 these haplotype new specific primers were designed and named AvPI-AF, AvPI-BF,
175 AvPI-CF, AvPI-DF and AvPI-EF respectively. These primers were used in our study to
176 screen for the presence of these haplotypes in other avian hosts using the PCR protocol
177 described above.

178 Phylogenetic analysis of the DNA sequences was performed using the “Maximum

179 Likelihood” calculation method and phylogenetic trees built with “Treeview” module
180 from BioEDIT® or PhyML module from Seaview 4 (GOUY *et al.* 2010).

181

182 **Results**

183

184 *Morphological study*

185 *Plasmodium* parasites could not be found in the blood smears or the tissue sets collected
186 from penguins, Fifteen of the twenty-six (58%) Eurasian Magpies and four of the nine
187 (44%) Carrion Crows had morphologically detectable *Plasmodium* parasites. Of these, all
188 had several distinguishable *Plasmodium* species in their blood. Several other blood
189 parasites were also noted (Table 1 and Table 2): *Parahaemoproteus* spp. in 20 magpies
190 (77%) and five crows (56%), *Trypanosoma* spp. in three magpies (12%) and five crows
191 (56%), *Leucocytozoon* spp. in four magpies (15%) and two crows (22%) and an
192 unidentified parasite in a crow (possibly a *Lankesterella* sp.).

193 Similarly to CHAVATTE *et al.* (2007), a high diversity of *Plasmodium* was recorded
194 with ten *Plasmodium* species recorded in Eurasian Magpies and six species in Carrion
195 Crows: *Plasmodium lenoblei* (11 magpies and one crow), *Plasmodium dorsti* (nine
196 magpies and three crows), *Plasmodium bioccai* (nine magpies and two crows),
197 *Plasmodium* sp.1 (seven magpies and two crows), *Plasmodium relictum* (four magpies
198 and four crows), *Plasmodium dherteeae* (five magpies), *Plasmodium beaucournui* (five
199 magpies), *Plasmodium maior* (four crows), *Plasmodium tranieri* (three magpies),
200 *Plasmodium* sp.2 (three magpies), and *Plasmodium snounoui* (one magpie). Two
201 *Plasmodium* species might potentially be new species but they can’t be described due to

202 insufficient material.

203

204 *Molecular study*

205 In the Eurasian Magpies and Carrion Crows

206 Positive amplification of 18 ssrRNA gene fragments was obtained for all crows and
207 magpies that were blood smear positive for *Plasmodium* spp. All crows and magpies in
208 which no *Plasmodium* spp. were detected by microscopic examination had negative PCR
209 results.

210 Seventeen sequences for the rPLU1-rPLU5 fragment were obtained from the corvids.
211 BLAST comparison to published sequences showed that our sequences clustered with 4
212 of the 5 types (A, B, C and E) previously recorded from the magpies in Paris' region
213 (Figure 1). They were named according to the type they corresponded, for some the
214 homology was complete, whereas for others limited sequence variations that might
215 correspond to subtypes was noted Table 4.

216 A representative sequence from each Types/Subtypes recorded in La Palmyre Zoo has
217 been deposited in GenBank (accession number: XXXXX to XXXXX).

218 The sequences isolated from the corvids showed differences ranging from 3.6 to 19.8%
219 from those previously observed in other avian *Plasmodium* species: *P. cathemerium*
220 (AY625607), *P. gallinaceum* (M61723), *P. lophurae* (X13706) and *P. juxtannucleare*
221 (AF463507, AF159790) (Table 4).

222

223 In the African Black-footed Penguins

224 Of the 44 penguins, we first used in duplicate a non optimized PCR protocol on the

225 primary blood sample collected for each penguin: we got 4 clearly positive results - *i.e.*
226 positive in both duplicate (penguins: 1597, 3817, 6373, and F142), 27 dubious results -
227 *i.e.* positive only in one of the duplicate with sometime only a very poorly marked band
228 and 13 negative results - *i.e.* negative in both duplicate (Table 3).

229 Given the high number of uncertain results that was probably due to the low parasite
230 burdens, an optimized protocol in which a higher volume of template (3 μ l and 2 μ l in the
231 primary and secondary amplifications respectively) and an increase in the number of
232 cycles (35 in both amplification reactions), was employed in duplicate. Using the
233 optimized protocol: the 4 positive penguins were confirmed positive in both duplicate; 23
234 of the dubious results were found positive in at least one of the duplicate with a better
235 marked band while the remaining 4 dubious results were found negative in both
236 duplicates; 1 negative results turned to be positive in both duplicates and the 12 others
237 negatives results were confirmed negative in both duplicates (Table 3).

238 A penguin was considered positive when at least two positive amplifications within the
239 four tests were obtained from a particular sample; therefore *Plasmodium* was detected in
240 a total of 28 penguins (Table 3). When an additional blood sample was available for a
241 penguin, it was analyzed as separate sample (Table 3) and we noticed that in the 4
242 positive penguins with blood samples collected on November 2006 and May 2007
243 parasite DNA was detected only in the samples collected in May.

244

245 In the 4 positive penguins (1597, 3817, 6373, and F142) screening for the specific Avian
246 *Plasmodium* Type was performed using the Type-specific primers. The four penguins
247 showed only infection by Type B parasites. Sequencing confirmation was performed on a

248 bigger fragment with another nt-PCR using the same conditions of reaction and the
249 primers pair rPLU3 and rPLU2 (ATCTAAGAATTTACCTCTGACATCTG) size
250 ~870bp. All the four sequences were confirmed similar to the Avian *Plasmodium* Type
251 B-1 detected in the European Magpies and Carrion Crows from La Palmyre Zoo (Figure
252 2). The sequence obtained from the penguin is deposited in GenBank under the accession
253 number XXXXX.

254

255 Discussion

256

257 The aim of this study was to evaluate current *Plasmodium* infections in penguins
258 undergoing medical prophylaxis with pyrimethamine at La Palmyre zoo, and try to
259 determine if two species of free-ranging pest avian species (*Pica pica* and *Corvus corone*)
260 could act as a reservoir of the infection.

261 These two corvids species showed high prevalences of *Haemosporidia* infections, and
262 hosted many parasite species belonging to the Genera *Plasmodium*, *Parahaemoproteus*,
263 and *Leucocytozoon*. The molecular study enabled confirmation of these infections : all
264 magpies and crows for which *Plasmodium* parasitemias were noted on blood smears gave
265 positive PCR results, and conversely no amplification was obtained in magpies and crows
266 that showed no *Plasmodium* parasitemias. The amplified DNA fragments were assembled
267 in different types and subtypes corresponding to those isolated by another study in Paris'
268 region (CHAVATTE *et al.* 2007). In the present state of our knowledge it is not possible
269 to determine a correspondence between these molecular types/subtypes and the species
270 identified by morphological analysis because *i) Plasmodium* classification relies on

271 morphological criteria and *ii*) polyparasitism was detected in all infected magpies and
272 crows similarly to [CHAVATTE *et al.* \(2007\)](#). Furthermore, it is known that such a
273 correspondence is hazardous and many parasite misidentifications have been reported in
274 GenBank ([VALKIŪNAS *et al.* 2008](#)).

275 On the 18S ssrRNA fragment considered in our study the percentage of variation with *P.*
276 *cathemerium* (AY625607) and *P. gallinaceum* (M61723) that belong to the *Haemamoeba*
277 subgenus is 4.5%. This percentage of variation rises to 7.7 – 8.1% when comparing with
278 *P. lophurae* (X13706) that belong to the *Giovannolaia* subgenus. Then it increases to
279 10.5 – 13% when comparing with the *P. juxtannucleare* (AF463507, AF159790) that
280 belongs to the *Bennettinia* subgenus. Among our sequences within a same “Type” the
281 percentage of variation varies between 0 – 3.2% while it can vary from 2.3 to 22.4%
282 between the different “Types”. Therefore it is possible to hypothesize that these
283 sequences characterized different species of *Plasmodium* some of which might belong to
284 different subgenera.

285 These percentages of variation combined with the phylogenetic study show that the
286 sequences from Types B and C seem closely related to each other and group with the
287 sequences from the *Haemamoeba* species, while the Type A sequence seems more related
288 to the *Bennettinia* subgenus. Regarding the sequences from Type E, they appear to be not
289 related to published ones and might belong to another subgenus like *Papernaia*
290 ([LANDAU *et al.* 2010](#)).

291 These observations added with the presence of several types/subtypes in each of the
292 infected magpies or crows corroborated the results of the morphological approach that
293 reported the presence of several species from different subgenera recently described from

294 the magpie in Paris' region and the constant polyparasitism by several species of
295 *Plasmodium* (CHAVATTE *et al.* 2007).

296 With a percentage of variation among our sequences reaching 22.4% and the concurrent
297 infection by several other haemosporidian parasites like *Parahaemoproteus* and
298 *Leucocytozoon* the exact nature of the amplified fragments might be questionable.

299 However the specificity of the oligonucleotide primers used in our study appears to be
300 restricted to *Plasmodium* parasites only. In fact when comparing the morphological
301 findings and the PCR results, we constantly obtained negative results from magpies and
302 crows infected by *Parahaemoproteus* and/or *Leucocytozoon* while we constantly obtained
303 positive results in those infected by *Plasmodium*. Similar observation has been made on
304 Haemosporidia from raptors in which parasite diversity was studied by 18S ssrRNA and
305 cyt-b gene concurrently (CHAVATTE pers. com.)

306 In penguins, PCR enabled detection of parasitic DNA in 28 specimens whereas no
307 penguin had shown detectable blood parasites on blood smears. PCR therefore showed a
308 higher sensitivity in that species, compared to the morphological study, and enabled
309 detection of low parasitemia infections, that could be chronic poorly pathogenic
310 infections, or a consequence of the medical prophylaxis carried out during our studies.

311 Nonetheless, 5 of the 28 penguins that gave positive PCR results were less than 1 year old
312 when blood samples were made, and chronic infections are unlikely in young penguins,
313 whose sensitivity to *Plasmodium* infections have been reported several times (GRIM *et*
314 *al.* 2003, PARSONS&UNDERHILL 2005, PETIT 1998, STOSKOPF & BEALL 1980).

315 Therefore the second hypothesis seems more likely.

316 Sequences obtained from penguins F142, 3817,1597, and 6373 are detectable with the

317 oligonucleotide primer AvPl-BF specific of the Avian *Plasmodium* Type B and show
318 exact similarity with the avian *Plasmodium* Type-B isolated from the magpies and crows.
319 This result confirms that these free-ranging birds and the *Spheniscus demersus* hosted at
320 La Palmyre Zoo share the same parasite. Unfortunately no parasites were observed in the
321 blood of the penguins and therefore no correlation between these molecular findings and
322 morphological considerations in terms of species/subspecies can be done.

323 In 4 of the 5 penguins with two blood samples (November 2006 and May 2007) DNA of
324 the parasite was detected only in May. This trend matches the previously reported
325 seasonality of *Plasmodium* infections in Penguins, most cases of which occur from May
326 to October, particularly when high temperatures are encountered (CRANFIELD *et al.*
327 1990, GRACKZYK *et al.* 1994c, GRINER 1974, 1983, PARSONS & UNDERHILL
328 2005, GRACKZYK *et al.* 1994d, FIX *et al.* 1988, CHITTY 2006, RODHAIN 1939),
329 which can be explained by the maximal activity of the vector and higher transmission of
330 the parasite.

331 We identified two free-ranging avian species (*Pica pica* and *Corvus corone*) that host
332 many *Plasmodium* species some of which show molecular identity to the DNA obtained
333 in penguins. These two species very likely act as a reservoir of the infection for the
334 penguins, which confirms a hypothesis involving magpies as a reservoir of penguin
335 *Plasmodium* infections in Marwell Zoo (HUGH-JONES 1999). Other wild avian species
336 reported to be the reservoir of penguin *Plasmodium* spp. infections include house
337 sparrow, robins and other small song birds at Baltimore Zoo (CRANFIELD *et al.*, 1990),
338 some of these birds (*i.e.* house sparrow, robins) are present in La Palmyre Zoo and might
339 act as reservoir as well, but to the authors' knowledge, no previous study has established

340 molecular evidence of parasite identity between a suspected avian reservoir species and
341 infected penguins.

342 The prevalence of *Plasmodium* spp. infections detected in the penguin colony at La
343 Palmyre Zoo is quite high (28/44 individuals, 64%). As shown in the morphological
344 study, no parasites were seen in the penguin blood smears : this means that infections
345 detected by PCR were weak (we indeed obtained several dubious results, with poorly
346 marked bands, although we used a very sensitive molecular test like nt-PCR). It could be
347 hypothesized that even weaker infections could exist, and not be detected by molecular
348 techniques. However, this would be surprising, knowing the great sensitivity of molecular
349 tools and the previously described high susceptibility of penguins to *Plasmodium*
350 infections. Another hypothesis could be the infection by a poorly pathogenic *Plasmodium*
351 species. Penguins' susceptibility to malaria was indeed reported only for *Plasmodium*
352 *relictum* (CRANFIELD *et al.* 1990, FLEISCHMAN *et al.* 1968a) GRACZYK &
353 CRANFIELD 1996, GRACZYK *et al.* 1994a, 1994b, 1994c, 1994d, GRINER 1974,
354 1983, JONES & SHELLAM 1999a, McCONKEY *et al.* 1996, PETIT 1998, RODHAIN
355 1938, 1939, RODHAIN & ANDRIANNE 1952, STOSKOPF & BEALL 1980,
356 STOSKOPF & BEIER 1979. CHITTY 2006, FIX *et al.* 1988, TOLLINI *et al.* 2000,
357 DUIGNAN 2001, PENRITH *et al.* 1994, REWELL 1948, BENNETT *et al.* 1992,
358 BROSSY 1993, BROSSY *et al.* 1999, FANTHAM & PORTER 1944, GRACZYK *et al.*
359 1995a, JONES & SHELLAM 1999b, LOMBARD *et al.* 1999, MILLER *et al.* 2001,
360 PARSONS & UNDERHILL 2005. GRACZYK *et al.* 1995b, LAIRD 1950), *Plasmodium*
361 *elongatum* (CRANFIELD *et al.* 1990, FIX *et al.* 1988, FLEISCHMAN *et al.* 1968a,
362 1968b, GRACZYK & CRANFIELD 1996, GRACZYK *et al.* 1994a, 1994b, 1994c,

363 1994d, HERMAN *et al.* 1968, JONES & SHELLAM 1999a, PETIT 1998, STOSKOPF
364 & BEALL 1980, STOSKOPF & BEIER 1979, TOLLINI *et al.* 2000), *Plasmodium*
365 *juxtannucleare* (GRIM *et al.* 2003), and *Plasmodium tejerai* (SILVEIRA *et al.* 2013)
366 infections. Yet these three species were rarely observed in our magpies and crows (only
367 *Plasmodium relictum* was observed, in 4 magpies and 4 crows). The virulence and
368 pathogenicity of a potential infection by *Plasmodium dorsti* (observed in 9 magpies and 3
369 crows), *Plasmodium lenoblei* (observed in 11 magpies and 1 crow), or *Plasmodium*
370 *bioccai* (observed in 9 magpies and 2 crows) in penguins are completely unknown
371 because these species, quite recently described (CHAVATTE *et al.* 2007) have never
372 been observed in *Sphenisciformes*.

373 In order to obtain a morphological identification of the incriminate species and to verify
374 these hypotheses, an experimental model using subinoculation of infected blood in a
375 susceptible host could be tried. Alternatively, systematic tests on naive, untreated penguin
376 in the colony could furnish some answers.

377 In addition, it is likely that the pyrimethamine prophylaxis undertaken during the study
378 has severely diminished the intensity of the parasitemias, making them “invisible” on
379 blood smears and hardly detectable by PCR, explaining the poorly marked bands and
380 dubious results we obtained.

381 Pyrimethamine is a slow acting inhibitor of the dihydrofolate reductase that is active on
382 the erythrocytic schizonts and has shown some effects on the exoerythrocytic schyzogony
383 and the sporogony (BRAY *et al.* 1959, WHO 2006). In human malaria, this drug is now
384 always used in synergistic treatment with slow acting sulfonamide (WHO 2006). Many
385 studies have focused on its pharmacokinetics and acting mode in human malarial

386 infection as well as in the experimental avian model *P. gallinaceum* in chicks (SINGH *et*
387 *al.* 1952, GREENBERG & BOND 1954, ROLLO 1955). Malaria prophylaxis in penguins
388 using pyrimethamine associated with sulfadiazine is one of the drug regimen endorsed by
389 the Penguin Taxon Advisory Group in the American Zoo and Aquarium Association
390 (AZA), with some caution linked to the teratogenic effect of the pyrimethamine, and
391 reported to be used in several institutions (WALLACE & WALSH 2005).

392 A serologic survey of the penguin colony could also teach us more about the
393 characteristics of the *Plasmodium* infections, and help us compare serological tools and
394 PCR, to determine the pros and cons of both diagnostic techniques in captive penguin
395 colonies.

396 Finally, we also lack a study of the vector activity in La Palmyre Zoo, to establish a
397 quantifiable link between corvids and penguins and learn more about the epidemiology of
398 the infection. *Plasmodium* and/or *Haemoproteus* infections in *Culex pipiens* and *Culex*
399 *torrentium* were recently detected by PCR at Basel Zoo, where *Plasmodium relictum*
400 infections were established for two *Spheniscus demersus* (CEREGHETTI *et al.* 2012).
401 Another study (McCONKEY *et al.* 1996) established molecular evidence of parasite
402 similarity between infected mosquitos and a lethal *Plasmodium relictum* infection in a
403 *Spheniscus demersus* at Baltimore Zoo. However a study showing molecular similarity of
404 *Plasmodium* spp. sequences in penguins, mosquitos and wild reservoir species of birds at
405 the same time, and at the same place is still lacking. Seeking potential preferences of
406 mosquitos for some avian species (corvids or penguins), quantifying the feeding behavior
407 of the females (number of blood meals per day, amount of blood ingested per meal, life
408 span) and estimating the prevalence of mosquito *Plasmodium* infections (seeking

409 sporozoites in salivary glands) are interesting prospects.

410

411 **References**

412

- 413 1. Bennett G.F., Earlé R.A., Du Toit H., Huchzermeyer F.W., 1992. A host-parasite
414 catalogue of the haematozoa of the sub-saharan birds. *Onderstepoort J. Vet. Res.*
415 59, 1-73.
- 416 2. Bray R.S., Burgess R.W., Fox R.M., Miller M.J., 1959. Effect of pyrimethamine
417 upon sporogony and pre-erythrocytic schizogony of *Laveriana falciparum*. *Bull.*
418 *Wld. Hlth. Org.* 21, 233-238.
- 419 3. Bray R.S., Garnham P.C.C., 1962. The Giemsa-colophonium method for staining
420 protozoa in tissue section. *Indian J. Malariol.* 16, 153-155.
- 421 4. Brossy J.J., 1992. Malaria in wild and captive Jackass penguins *Spheniscus*
422 *demersus* along the southern African coast. *Ostrich* 63, 10-12.
- 423 5. Brossy J.J., 1993. Haemoparasites in the African (Jackass) Penguin (*Spheniscus*
424 *demersus*). *Penguin Conservation.* 11, 20-21.
- 425 6. Brossy J.J., Plös A.L., Blackbeard J.M., Kline A., 1999. Diseases acquired by
426 captive penguins: what happens when they are released into the wild? *Marine*
427 *Ornithology.* 27, 185-186.
- 428 7. Cereghetti N., Wenker C., Hoby S., Müller P., Marti H., Lengeler C., 2012. The
429 mosquito project – vector-based avian malaria prevention strategies in the Zoo
430 Basel. *Proc. Int. Conf. Dis. Zoo Wild Anim.* p 75.

- 431 8. Chavatte J.-M., Chiron F., Chabaud A.G., Landau I., 2007. Fidélisation du couple
432 hôte-vecteur facteur probable de spéciation: 14 espèces de *Plasmodium* de la Pie.
433 *Parasite*. 14, 21-37.
- 434 9. Chitty J., 2006. Marwell zoo, U.K. *International Zoo News*. 53/8, 492-493.
- 435 10. Cranfield M.R., Shaw M., Beall F., Skjoldager M., Ialeggio D., 1990. A review
436 and update of avian malaria in the African penguin (*Spheniscus demersus*). *Proc.*
437 *Amer. Assoc. Zoo Vet.* 21, 243-248.
- 438 11. Duignan P.J., 2001. Diseases of penguins. *Surveillance*. 28, 5-11.
- 439 12. Fantham H.B., Porter A., 1944. On a *Plasmodium* (*Plasmodium relictum* var.
440 *spheniscidae* n. var.) observed in four species of penguins. *Proc. Zool. Soc. Lond.*
441 114, 279-292.
- 442 13. Fix A.S., Waterhouse C., Greiner E.C., Stoskopf M.K., 1988. *Plasmodium*
443 *relictum* as a cause of avian malaria in wild-caught Magellanic penguins. *J. Wildl.*
444 *Dis.* 24, 610-619.
- 445 14. Fleischman R.W., Squire R.A., Sladen W.L.J., Melby Jr E.C., 1968a. Malaria
446 (*Plasmodium elongatum*) in captive African penguins (*Spheniscus demersus*). *J.*
447 *Amer. Vet. Med. Assoc.* 153, 928-935.
- 448 15. Fleischman R.W., Squire R.A., Sladen W.L.J., Moore J., 1968b. Pathologic
449 confirmation of malaria (*Plasmodium elongatum*) in African penguins
450 (*Spheniscus demersus*). *Bull. Wildl. Dis. Assoc.* 4, 133-135.
- 451 16. Gouy M., Guindon S., Gascuel O., 2010. SeaView Version 4: A Multiplatform
452 Graphical User Interface for Sequence Alignment and Phylogenetic Tree
453 Building. *Mol. Biol. Evol.* 27, 221-224.

- 454 17. Graczyk T.K., Cranfield M.R., 1996. A model for the prediction of relative titers
455 of avian malaria and *Aspergillus* spp. IgG in Jackass penguin (*Spheniscus*
456 *demersus*) females based on maternal IgG in egg-yolk. *International Journal for*
457 *Parasitology*. 26, 749-754.
- 458 18. Graczyk T.K., Brossy J.J., Plos A., Stoskopf M.K., 1995a. Avian malaria
459 seroprevalence in Jackass penguins (*Spheniscus demersus*) in South Africa. *J.*
460 *Parasitol.* 81, 703-707.
- 461 19. Graczyk T.K., Cockrem J.F., Cranfield M.R., Darby J.T., Moore P., 1995b. Avian
462 malaria seroprevalence in wild New Zealand penguins. *Parasite*. 2, 401-405.
- 463 20. Graczyk T.K., Cranfield M.R., McCutchan T.F., Bicknese E.J., 1994a.
464 Characteristics of naturally acquired avian malaria infections in naive juvenile
465 African black-footed penguins (*Spheniscus demersus*). *Parasitol. Res.* 80, 634-
466 637.
- 467 21. Graczyk T.K., Cranfield M.R., Shaw M.L., Craig L.E., 1994b. Maternal
468 antibodies against *Plasmodium* spp. in African black-footed penguins (*Spheniscus*
469 *demersus*) chicks. *J. Wildl. Dis.* 30, 365-371.
- 470 22. Graczyk T.K., Cranfield M.R., Skjoldager M.L., Shaw M.L., 1994c. An ELISA
471 for detecting anti-*Plasmodium* spp. antibodies in African black-footed penguins
472 (*Spheniscus demersus*). *J. Parasitol.* 80, 60-66.
- 473 23. Graczyk T.K., Shaw M.L., Cranfield M.R., Beall F.B., 1994. Hematologic
474 characteristics of avian malaria cases in African black-footed penguins
475 (*Spheniscus demersus*) during the first outdoor exposure season. *J. Parasitol.* 80,
476 302-308.

- 477 24. Greenberg J., Bond H.W., 1954. Resistance of a Pyrimethamine-Resistant strain
478 of *Plasmodium gallinaceum* to certain 2,4-Diaminopyrimidines and related
479 compounds. *J. Parasitol.* 40, 472-475.
- 480 25. Grim K.C., Van Der Merwe E., Sullivan M., Parsons N., McCutchan T.F.,
481 Cranfield M., 2003. *Plasmodium juxtannucleare* associated with mortality in black-
482 footed penguins (*Spheniscus demersus*) admitted to a rehabilitation center. *J. Zoo*
483 *Wildl. Med.* 34, 250-255.
- 484 26. Griner L.A., 1974. Some diseases of zoo animals. *Advances in Veterinary Science*
485 *and Comparative Medicine.* 18, 251-271.
- 486 27. Griner L.A., 1983. *Pathology of zoo animals – A review of necropsies conducted*
487 *over a fourteen-year period at the San Diego Zoo and San Diego Wild Animal*
488 *Park.* San Diego: Zoological Society of San Diego, 608p.
- 489 28. Hugh-Jones M. 1999. ProMED-mail – Malaria, zoo penguins – UK. *In: 09-OCT-*
490 *1999, 19991009.1805* [online], ISID [<http://www.promedmail.org>] (consulted 07
491 November 2007).
- 492 29. Jones H.I., Shellam G.R., 1999a. Blood parasites in penguins, and their potential
493 impact on conservation. *Marine Ornithology.* 27, 181-184.
- 494 30. Jones H.I., Shellam G.R., 1999b. The occurrence of blood-inhabiting protozoa in
495 captive and free-living penguins. *Polar Biol.* 21, 5-10.
- 496 31. Laird M., 1950. Some blood parasites of New-Zealand birds. *Zoology*
497 *Publications, Victoria University College.* 5, 1-20.
- 498 32. Landau I., Chavatte J.-M., Peters W., Chabaud A.G., 2010 The sub-genera of
499 avian *Plasmodium*. *Parasite.* 17, 3-7.

- 500 33. Leclerc A., 2008. Infections à *Plasmodium* chez les oiseaux Sphénisciformes
501 (manchots) ; synthèse bibliographique, analyse rétrospective de cas et étude
502 épidémiologique au zoo de La Palmyre. Veterinary thesis, Ecole Nationale
503 Vétérinaire d'Alfort, 284p.
- 504 34. Lombard E., Brossy J.J., Blackbeard J., 1999. Malaria in an African penguin.
505 *British J. Heamatol.* 106, 577.
- 506 35. McConkey G.A., LI J., Rogers M.G., Seeley D.C., Graczyk T.K., Cranfield M.R.,
507 McCutchan T.F., 1996. Parasite diversity in an endemic region for avian malaria
508 and identification of a parasite causing penguin mortality. *J. Euk. Microbiol.* 43,
509 393-399.
- 510 36. Miller G.D., Hofkin B.V., Snell H., Hahn A., Miller R.D., 2001. Avian malaria
511 and Marek's disease: potential threats to Galapagos penguins *Spheniscus*
512 *mendiculus*. *Marine Ornithology.* 29, 43-46.
- 513 37. Parsons N.J., Underhill L.G., 2005. Oiled and injured African penguins
514 *Spheniscus demersus* and other seabirds admitted for rehabilitation in the Western
515 Cape, South Africa, 2001 and 2002. *African J. Marine Science.* 27, 289-296.
- 516 38. Penrith M.L., Huchzermeyer F.W., De Wet S.C., Penrith M.J., 1994. Concurrent
517 infection with *Clostridium* and *Plasmodium* in a captive king penguin,
518 *Aptenodytes patagonicus*. *Avian Pathology.* 23, 373-380.
- 519 39. Petit T., 1998. Penguin malaria survey. In: *EEP Yearbook 1996/1997 including*
520 *the Proceedings of the 14th EAZA/EEP Conference.* Alphen a/d Rijn, 8-12
521 October 1997. Amsterdam : EAZA Executive Office, 481-482.

- 522 40. Rewell R.E., 1948. Report of the pathologist for the year 1947. *Proc. Zool. Soc.*
523 *Lond.* 118, 501-514.
- 524 41. Rodhain J., 1937. Une infection à *Plasmodium* chez *Spheniscus demersus*
525 (Manchot du Cap). *Annales de Parasitologie.* 15, 253-258.
- 526 42. Rodhain J., 1938. Schizogonie sans pigment chez les pingouins infectés de
527 *Plasmodium praecox (relictum)*. *C.R. de la Soc. de Biol.* 127, 368-372.
- 528 43. Rodhain J., 1939. L'infection à *Plasmodium relictum* chez les pingouins. *Annales*
529 *de Parasitologie.* 17, 139-157.
- 530 44. Rodhain J., Andrianne V.F., 1952. Deux nouveaux cas d'infestation par
531 *Plasmodium* chez les pingouins. *Annales de Parasitologie.* 27, 573-577.
- 532 45. Scott H., 1927. Report on the deaths occurring in the Society's Gardens during the
533 year 1926. *Proc. Zool. Soc. Lond.* 97, 173-198.
- 534 46. Silveira P., Belo N.O., Iacorte G.A., Kolesnikovas C.K., Vanstreels R.E., Steindel
535 M., Catão-Dias J.L., Valkiūnas G., Braga E.M., 2013. Parasitological and new
536 molecular-phylogenetic characterization of the malaria parasite *Plasmodium*
537 *tejerai* in South American penguins. *Parasitol. Int.* 62, 165-171.
- 538 47. Singh J., Basu P.C., Ray A.P., 1952. Screening of antimalarials against *P.*
539 *gallinaceum* in chicks. *Indian J. Malariol.* 6, 145-158.
- 540 48. Snounou G., Viriyakosol S., Jarra W., Thaithong S., Brown K.N., 1993.
541 Identification of the four human malaria parasite species in field samples by the
542 polymerase chain reaction and detection of a high prevalence of mixed infections.
543 *Mol. Biochem. Parasitol.* 58, 283-292.

- 544 49. Stoskopf M.K., Beall F.B., 1980. The husbandry and medicine of captive
545 penguins. *Proc. Amer. Assoc. Zoo Vet.* 11, 81-96
- 546 50. Stoskopf M.K., Beier B.A., 1979. Avian malaria in African black-footed
547 penguins. *J. Amer. Vet. Med. Assoc.* 175, 944-947.
- 548 51. Tollini J., Brocksen A., Sureda N., 2000. Prevention and treatment of avian
549 malaria in a captive penguin colony. *Penguin Conservation.* 13, 28-31.
- 550 52. Valkiūnas G., Atkinson C.T., Bensch S., Sehgal R.N.M., Ricklefs R.E. 2008.
551 Parasite misidentifications in GenBank: how to minimize their number? *Trends*
552 *Parasitol.* 24, 247-248.
- 553 53. Wallace R., Walsh M., 2005. Chapter 6: Health – Malaria, *in* Penguin Husbandry
554 Manual – 3rd Editions, *American Zoo and Aquarium Association.* 90-91.
- 555 54. World Health Organization, 2006. Guidelines for the treatment of malaria. WHO
556 Press, Geneva, Switzerland, 254pp.
- 557

558 **Tables**
 559

560 Table 1: Blood parasites observed on the blood smears prepared from the Eurasian

561 Magpies (*Pica pica*) captured at La Palmyre Zoo

562

Magpie ID	<i>Plasmodium</i> spp.	<i>Parahaemoproteus</i> spp.	<i>Leucocytozoon</i> spp.	<i>Trypanosoma</i> spp.	Others
1	+	++	-	-	-
2	+	++	-	-	-
3	+	++	-	-	-
4	++/+	+/+	-	-	-
5	+/+	++/+	-	-	-
6	-	++	-	-	-
7	+	++	-	-	-
8	-	++	-	-	-
9	+/++	+/++	-	+	-
10	-	-	-	-	-
11	-	-	-	-	-
12	++/++	++++++/+++	+/+	+	-
13	-	-	-	-	-
14	++/++	+/+	-	-	-
15	-	-	-	-	-
16	-	-	-	-	-
17	-	-	-	-	-
18	+	++	-	-	-
19	++	+	-	-	-
20	-	++++++	-	-	-
21	+++	++	++	+	-
22	+	++	-	-	-
23	+	+	-	-	-
24	+	++	+	-	-
25	-	++	-	-	-
26	-	+	+	-	-

563 *ID: identification number. + or -: presence or absence of blood parasites*
 564 *(morphological analysis). Parasitaemias were semi-quantitatively scored: the number of*
 565 *« + » symbols is proportional to the intensity of the parasitaemia. Several results in a*
 566 *single cell, separated by the « / » symbol indicate several blood smears for the same*
 567 *individual collected on different dates.*

568 Table 2: Blood parasites observed on the blood smears prepared from the Carrion Crows
 569 (*Corvus corone*) captured at La Palmyre Zoo

570

Carrion Crow ID	<i>Plasmodium</i> spp.	<i>Parahaemoproteus</i> spp.	<i>Leucocytozoon</i> spp.	<i>Trypanosoma</i> spp.	Autres
1	+	+	-	-	-
2	++	+	-	+	-
3	++	+	-	+	-
4	-	++++	+	-	-
6	-	-	-	-	<i>Lankesterella</i> ?
7	-	-	-	+	-
8	-	-	-	+	-
9	-	-	+	-	-
10	+	+	-	+	-

571 *ID: identification number. + or -: presence or absence of blood parasites*

572 *(morphological analysis). Parasitaemias were semi-quantitatively scored: the number of*

573 *« + » symbols is proportional to the intensity of the parasitaemia.*

Penguin ID	Sample date		Molecular analysis				Conclusion on the 4 replicates
			non optimized protocol		optimized protocol		
	EDTA blood primary sample	EDTA blood additional sample	1 st replicate	2 nd replicate	1 st replicate	2 nd replicate	
8976	04/05/2007		-	+	+	+	Pos
D18C	04/05/2007		(+)	-	+	+	Pos
3817	04/05/2007	16/11/2006	+	+	+ / [-]	+ / [-]	Pos
3895	04/05/2007		+	-	+	+	Pos
F142	04/05/2007		+	+	+	+	Pos
1597	04/05/2007	16/11/2006	+	+	+ / [-]	+ / [-]	Pos
5952	04/05/2007	12/07/2007	-	-	- / [-]	- / [-]	Neg
4212	04/05/2007		-	-	-	-	Neg
6373	04/05/2007	16/11/2006	+	+	+ / [-]	+ / [-]	Pos
6CEO	04/05/2007		-	+	+	+	Pos
2047	04/05/2007	16/08/2007	-	(+)	+ / [-]	+ / [-]	Pos
5401	04/05/2007			(+)			Neg
4F26	04/05/2007		(+)	-	-	-	Neg
F4F6	04/05/2007		-	-	-	-	Neg
041B	04/05/2007		-	(+)	-	+	Pos
OA1F	04/05/2007		-	(+)	+	+	Pos
7307	04/05/2007		-	(+)	+	+	Pos
8878	04/05/2007		-	-	+	+	Pos
2077	04/05/2007		+	-	+	+	Pos
28ED	04/05/2007		-	-	-	-	Neg
EC20	04/05/2007		(+)	-	+	+	Pos
3130	04/05/2007		(+)				Neg
3866	04/05/2007		-	-	-	-	Neg
BA14	04/05/2007		-	-	-	-	Neg
1532	04/05/2007		-	-	-	-	Neg
F691	04/05/2007		-	+	-	+	Pos
7292	04/05/2007		-	(+)	-	+	Pos
2582	04/05/2007		(+)	-	-	-	Neg
5545	04/05/2007	16/11/2006	-	-	- / [-]	- / [-]	Neg
7753	04/05/2007		(+)	-	+	+	Pos
86BB	04/05/2007		+	-	-	+	Pos
OO16	04/05/2007		(+)	-	+	+	Pos
093E	04/05/2007		(+)	-	-	+	Pos
2759	04/05/2007		-	-	-	-	Neg
CF51	04/05/2007		-	(+)	-	+	Pos
4624	04/05/2007		-	+	+	+	Pos
2784	04/05/2007		(+)	-	-	+	Pos
5916	04/05/2007		-	-	-	-	Neg

E1CC	04/05/2007		–	(+)	+	+	Pos
3071	04/05/2007		+	–	+	+	Pos
OD69	04/05/2007		–	–	–	–	Neg
7264	04/05/2007	16/11/2006	(+)	–	+ / [–]	+ / [–]	Pos
9102	25/07/2007		–	–	–	–	Neg
6601	25/07/2007		(+)	–	+	+	Pos

Table 3: Results table of the molecular assays performed on the penguins' blood samples. The non optimized protocol used at first gave 4 positives results (*i.e.* positive in both replicates), 27 dubious results (*i.e.* positive in one replicate only with poorly marked band) and 13 negative results (*i.e.* negative in both replicates). The optimized protocol gave 21 positive results (*i.e.* positive in both replicates); 7 dubious results (*i.e.* positive in one replicate only) and 16 negative results (*i.e.* negative in both replicates). While combining the results of the 4 replicates and considering a penguin to be positive when results are positive in at least 2 replicates we obtained 28 positive animals and 16 negative. «+ or –» for positive or negative results respectively; results in bracket indicate very poorly marked band; results on additional sample when available are reported in square bracket.

Table 4:

AvPI-TypeB_[KF061242]	La Palmyre-TypeB-1	La Palmyre-TypeB-3	La Palmyre-TypeB-2	AvPI-TypeC_[KF061243]	La Palmyre-TypeC-1	La Palmyre-TypeC-2	<i>P. cathemerium</i> _[AY625607]	<i>P. gallinaceum</i> _[M61723]	La Palmyre-TypeE-2	AvPI-TypeE_[KF061245]	La Palmyre-TypeE-1	<i>P. lophurae</i> _[X13706]	AvPI-TypeD_[KF061244]	<i>P. juxtannucleare</i> _[AF159790]	<i>P. juxtannucleare</i> _[AF463507]	AvPI-TypeA_[KF061241]	La Palmyre-TypeA
100.0																	
97.8	97.8																
97.6	97.6	99.0															
95.5	95.5	96.6	97.5														
95.5	95.5	96.8	97.7	99.6													
95.3	95.3	96.7	97.3	99.2	99.4												
95.3	95.3	96.4	96.3	94.4	94.6	94.3											
94.2	94.2	95.4	95.3	93.8	93.9	93.7	95.5										
94.1	94.1	95.5	95.2	93.8	94.0	94.0	93.7	93.7									
91.9	91.9	92.6	92.3	91.1	91.3	91.3	91.4	92.2	96.2								
93.8	93.8	94.6	94.3	93.1	93.3	93.5	92.9	93.6	98.4	97.2							
91.9	91.9	92.8	92.5	91.3	91.4	91.3	91.9	92.3	92.1	91.2	91.6						
85.4	85.4	86.8	87.2	87.2	87.3	87.0	85.4	84.9	86.4	83.6	85.9	83.6					
89.4	89.4	89.5	89.3	88.5	88.5	88.3	88.7	89.5	90.0	89.1	89.8	89.2	81.1				
88.2	88.2	88.0	88.0	88.0	87.9	87.7	87.0	87.2	88.7	88.1	88.7	88.2	80.2	94.2			
87.0	87.0	85.5	85.3	85.3	85.1	85.1	84.8	85.0	86.3	85.5	86.3	85.2	77.7	85.5	85.4		
86.9	86.9	85.3	85.2	85.2	85.0	85.0	84.6	84.9	86.1	85.4	86.2	85.1	77.6	85.4	85.4	99.9	

Comparison Table: Similarity percentages between the 18S ssrRNA sequences isolated from the Corvids in La Palmyre Zoo and published sequences from the Magpies' region (KF061241 to KF061245), *P. cathemerium* (AY625607), *P. gallinaceum* (M61723), *P. lophurae* (X13706) and *P. juxtannucleare* (AF463507, AF159790).

Figure captions

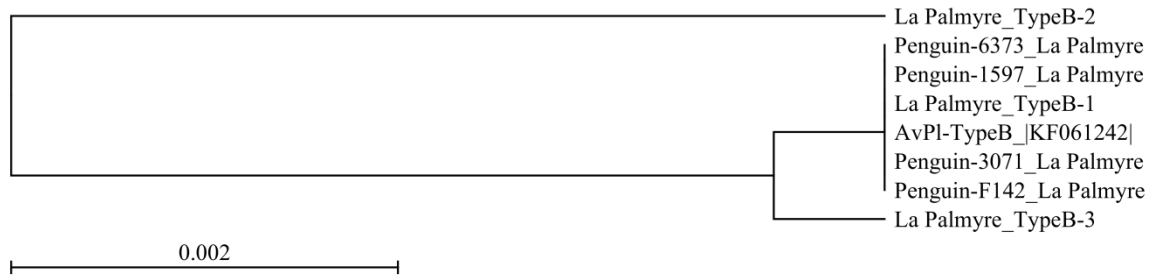
Figure 1:



Phylogenetic reconstruction based on the 18S rRNA and generated by the Maximum Likelihood (ML) method between *Plasmodium* species spread among Mammals, Avian and Reptilian hosts. Sequences length has been adjusted to allow comparison with *P. juxtannucleare* and *Plasmodium* from reptiles. The sequences isolated from the corvids in La Palmyre Zoo are highlighted in yellow and clustered with the sequences published

from the Magpie of the Paris' region. The Avian *Plasmodium* Type A seems related to *P. juxtannucleare*, while the Avian *Plasmodium* Type B and C form a clade with *P. gallinaceum* and *P. cathemerium*, then the Avian *Plasmodium* Type D and E form a separate clade.

Figure 2:



Phylogenetic reconstruction generated by ML method and based on the Avian *Plasmodium* Type B (fragment: rPLU3-rPLU2) including the published sequence and sequences obtained from Penguins and Corvids from La Palmyre Zoo. The sequences detected in the Penguins are exactly similar to one of those isolated from the Corvids of the same location as well as the published one from the Paris' region Magpie.