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4 **Prevalence of hematozoan parasites in Red Kite nestlings**
5 **from France**

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19

Abstract

21 Blood pathogens may be harmful for birds and thus, represent a potential threat for
22 some populations. To date, only few data are available on the prevalence of
23 hematozoans in the Red Kite, a raptor endemic to the Western Palearctic listed as
24 Near Threatened within its global range. Here, we report the first screening of
25 haemosporidian parasites in Red Kite nestlings from French breeding areas. The
26 blood of 47 nestlings was sampled in three areas in Eastern France and the
27 prevalence of three genera of blood pathogens, *Leucocytozoon*, *Haemoproteus* and
28 *Plasmodium*, was measured based on PCR-assays. Thirteen nestlings were positive
29 to *Leucocytozoon sp.* (n=13) and *Plasmodium relictum* (n=1), which corresponds to
30 an overall prevalence of 28%. This prevalence is higher than those previously
31 reported for the Red Kite in other countries. This is the first record of *P. relictum*, a
32 potential pathogen for birds, in the Red Kite. No relationship was found between the
33 body condition of nestlings and the occurrence of haemosporidians. Our findings
34 suggest that hematozoans could be a limited threat for nestlings but prevalence in
35 adults, a sensitive stage for the population dynamic of Red Kite, remains poorly
36 known and should be further investigated at least in its main breeding areas.

37

38 Key words: Haemosporida, *Haemoproteus*, *Leucocytozoon*, *Plasmodium*, raptor

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49 conservation plan.

50 All the authors consent to participate and for publication

51 Availability of data and material: The data presented in this article are referenced in
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56 and Michael Coeurdassier managed and carried out fieldwork and sampling. Eve
57 Afonso and Nicolas Bassin conducted the pathogen analysis. Michael Coeurdassier,
58 Eve Afonso, Thibaut Powolny and Nicolas Bassin analyzed and interpreted the data
59 and wrote the manuscript. All co-authors commented on the manuscript and
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72 they were performed.

73

74 Introduction

75 The Red Kite *Milvus milvus* is an Accipitridae endemic to the Western Palearctic. It
76 breeds mainly in Germany, France and Spain, which together hold around 75% of
77 the global population (Knott et al. 2010). In these countries, the populations have
78 been estimated to be declining, which led the International Union for the
79 Conservation of Nature to assess the Red Kite as Near threatened (BirdLife
80 International 2020) within its global range while it is currently listed as vulnerable in
81 France (UICN France et al. 2016). The main threats identified are poisoning by
82 anticholinesterasic pesticides and rodenticides, decrease of livestock,
83 homogenization of landscapes and potentially wind turbines (BirdLife International
84 2020). In both Europe and France, the decline reported during the period 1990-2010
85 has led to the implementation of a Conservation Plan in which the health status of
86 the Red Kite was surveyed, notably on nestlings that were caught at nests for
87 ringing and wing-marking. Among the health indicators monitored and considering
88 that ecotoxicological issues were identified, chemical concentrations in blood were
89 measured and we showed that 50% of nestlings were exposed to organic
90 compounds and/or metals (Coourdassier et al. 2014; Powolny et al. 2020). If
91 infection by pathogens has not been pointed out as a threat for the Red Kite, only
92 few studies have focused on the prevalence of blood parasites and no data are
93 available on the prevalence of hematozoan pathogens in Red Kite nestlings from
94 France. Haemosporidians belonging to three genera of protozoan (*Haemoproteus*,
95 *Leucocytozoon*, and *Plasmodium*) are intracellular blood parasites transmitted to
96 hosts by bloodsucking dipteran vectors, they have been commonly detected in birds
97 including raptors (Remple 2004). *Haemoproteus* and *Leucocytozoon* are often
98 considered to have little or no pathogenic effects on birds but their presence in some
99 raptor nestlings has been related to subclinical effects and even host death.
100 *Plasmodium*, the causative agent of malaria in animals, can be a serious avian

101 pathogen, *Plasmodium relictum* being the species most commonly associated with
102 virulent disease (Remple 2004). In raptors, haemosporidians may also negatively
103 affect health such as body condition and life traits (Korpimaki et al. 1993; Hatchwell
104 et al. 2001; Sol et al. 2003; Garvin et al. 2006). Here, we aim to measure the
105 prevalence of common blood parasites in birds of the genera *Haemoproteus*,
106 *Leucocytozoon* and *Plasmodium* in nestlings of three areas monitored in Eastern
107 France. The relationship between prevalence and body condition of the nestlings
108 was also checked to document whether such infection could affect individuals.

109 **Methods**

110 **Study areas and sample collection**

111 Fieldwork was carried out in 2015 from 4th to 25th June in Franche-Comté region,
112 Eastern France. Three breeding populations of Red Kite, from three areas (called
113 “Area” factor hereafter) were monitored: Sundgau Belfortain (47°380170’N,
114 6°510460’E), Dugeon-Remoray basin (46°540220’N, 6°210170’E) and Besançon
115 plateau (47°140350’N, 6°010190’E). Forty-seven chicks (23 males and 24 females)
116 corresponding to 24 nests (called “Nest” factor hereafter) were retained for the
117 study. The sampling was implemented 15 days prior to the expected fledging date,
118 when the chicks were approximately 25–40 days old. Nestlings were removed from
119 the nests by an experienced arborist climber and carried to the ground for data
120 collection. First, the body mass (g) and the wing length (i.e., length from the
121 extremity of the longest primary feather to the wrist of the right wing in mm) were
122 measured. Blood (2 mL) was sampled by trained operators from the brachial vein
123 using lithium-heparin coated syringes. As recommended (Fair et al. 2010), the
124 volume of blood collected was <0.5% of the chick’s body mass. Blood samples were
125 kept in the dark at 4°C and then stored at -20°C until processed in the laboratory.
126 The sex of the chicks was determined according to a PCR method (Griffiths et al.

127 1998) as described in Powolny et al. (2020). All the manipulations were performed
128 with governmental authorizations no. 2015093-0007 and 2015093-0023 in the
129 framework of the Red Kite national conservation plan.

130 **Body condition estimate**

131 In the present study, we define body condition as a proxy of energy reserves, mainly
132 fat mass that represents a broad indicator of the health status of nestlings.
133 According to Labocha and Hayes (2012), body mass of birds is « *as good or nearly*
134 *as good an indicator of fat content, as any of the other condition indices* ». Because
135 body mass can be easily and reliably measured, we used it as an estimate of body
136 condition. As we studied growing individuals, difference in body mass could be due
137 to slight differences in specimen age rather than in fat reserve. Mougeot and
138 Bretagnolle (2006) showed that the wing length was closely correlated to the age of
139 Red Kite nestlings. Moreover, the relationship they established is almost linear
140 within the range of ages of the kite chicks we studied, thus, we used the wing length
141 as an estimate of chick age. Body condition was calculated as the residuals of the
142 linear model [body mass (in g) ~ wing length (in mm)], which corresponds to an
143 assessment of the body mass following a statistical elimination (sensus Grafen and
144 Hails 2002) of age differences. Positive residuals were related to chicks in relative
145 good condition while negative residuals represented kites in poor condition.

146 **DNA extraction, PCR amplification, sequencing, and parasite detection**

147 DNA was extracted from blood samples using the DNeasy Blood & Tissue kit
148 (Qiagen, France) and according to manufacturer's instructions (protocol "Purification
149 of Total DNA from Animal Blood or Cells"). A nested PCR was conducted to amplify
150 a fragment of cytochrome b gene (542 bp) from species of *Plasmodium*,
151 *Haemoproteus* and *Leucocytozoon* as recommended by Pérez-Rodríguez et al.
152 (2013). A first PCR was performed in total volumes of 12.5µL including 1 × Accustart

153 II PCR ToughMix (Quanta Biosciences, USA), 0.6 mM of each primer
154 (Plas1F/HaemNR3), and 22 ng of total genomic DNA. The PCR program consisted
155 in an activation step of 3 min at 94°C, followed by 20 cycles of 30 sec at 94°C, 20
156 sec at 52°C, 30 sec at 72°C, and a final elongation of 5 min at 72°C. We then used
157 PCR products from the first PCR to conduct the following reaction in a total volume
158 of 25µL comprising 1 × Accustart II PCR ToughMix, 0.6 mM of each primer
159 (3760F/HaemJR4) and 2.5µL of PCR products from the first PCR. The PCR
160 program consisted in an activation step of 3 minutes at 94°C, followed by 35 cycles
161 of 30 sec at 94°C, 20 sec at 52°C, 30 sec at 70°C, and a final elongation of 5 min at
162 70°C. PCR products were then separated and visualized using a Qiaxcel device
163 (Qiagen, France) and a DNA High Resolution kit (Qiagen). A negative control (PCR
164 grade water instead of DNA extract) was introduced every ten samples. PCR
165 products which yielded an approximately 590 bp fragment (including primers) were
166 confirmed by sequencing. The amplified products were purified using the QIAquick
167 PCR Purification Kit (Qiagen) according to manufacturer's instructions. Direct
168 sequencing of the purified PCR products was performed with an automated
169 sequencer (Applied Biosystems 3130 Genetic Analyzer) using the primers employed
170 for the second PCR reactions. For each PCR-positive sample, we aligned forward
171 and reverse DNA sequences to obtain a consensus of 479 bp (Pérez-Rodríguez et
172 al. 2013). A homology search of the sequences generated in this study was then
173 performed by conducting an online search with the NCBI Basic Local Alignment
174 Search Tool for nucleotides (BLASTN) in the GenBank database
175 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

176 Mixed infections were checked by examining electropherograms using the software
177 BioEdit following the recommendations of Pérez-Tris and Bensch (2005). When
178 double peaks were observed, we compared double peak patterns with the DNA
179 sequences of parasite haplotypes already available on Genbank to identify which

180 parasite haplotypes were involved in mixed infections (Pérez-Rodríguez et al.,
181 2013).

182 **Data analysis**

183 Difference of infection status (i.e., presence/absence of hematozoans) between sex
184 was checked with a binomial generalized linear mixed effect model (GLMER) with
185 'Sex' as fixed factors and 'Nest' nested into 'Area' (Area/Nest) as random effects
186 [corresponding full model: Status ~ Sex + (1|Area/Nest)]. The relationship between
187 the presence of at least one positive chick in the brood ('Positive brood', binomial
188 variable) and the brood size was tested with a binomial generalized linear mixed
189 effect model (GLMER) with 'Brood size' as fixed factors and 'Area' as random
190 effects [corresponding full model: Positive brood ~ Brood size + (1|Area)].

191 Relationship between body condition and infection status of the individual was
192 checked with a linear mixed effect model (LMER) with 'Wing length', 'Sex' (in
193 interaction or not with 'Wing length') and 'Status' as fixed factors and 'Nest' nested
194 into 'Area' (Area/Nest) as random effects [corresponding full model: Body mass ~
195 Wing length + Sex + Status + (1|Area/Nest)] (Zuur et al., 2009). The null model was
196 also tested and the most parsimonious model with the lowest Akaike information
197 criterion (AICc) was selected, a difference of AICc > 2 was retained to evidence
198 difference between 2 models (Burnham and Anderson, 2010). Normality of model
199 residuals and variance homoscedasticity were verified. All statistical analyses were
200 performed using R 3.4.2. with the packages *lme4*, *pgirmess* and *lmerTest*.

201

202 **Results**

203 Among the 47 chicks studied, 30 came from the Dugeon-Remoray basin (15
204 females and 15 males), 14 from the Besançon plateau (eight females and six males)
205 and three from the Sundgau belfortain (one female and two males). Haemosporidian

206 parasites were found in 13 chicks (i.e., 28%), *Leucocytozoon* was detected in eight
207 males and five females from the three studied areas and *Plasmodium* in a single
208 male also infected by *Leucocytozoon* of the Sundgau belfortain population (Fig. 1).
209 No difference of *Leucocytozoon* prevalence was evidenced between sex (binomial
210 GLMER, $p = 0.33$) or between brood size (binomial GLMER, $p = 0.66$). We found
211 two *Leucocytozoon* haplotypes, which perfectly matched (i.e. with a 100% identity
212 on 479bp) with L-MILVUS1 (Genbank accession number JN164716) and L-
213 MILANS4 (JN164713) haplotypes described in Pérez-Rodríguez et al. (2013). The
214 DNA sequence corresponding to L-MILVUS1 also closely matched with
215 *Leucocytozoon toddi* (GenBank accession number DQ177272) with a 99% identity.
216 The DNA sequence amplified in the only sample positive for *Plasmodium* matched
217 with *Plasmodium relictum* (GenBank accession number LN835311) with a 100%
218 identity over 462bp. Although *Leucocytozoon toddi* is a typical parasite of birds
219 (Valkiūnas et al. 2010), we cannot exclude that we detected an unreferenced
220 species genetically close, we thus retained *Leucocytozoon* ssp. hereafter, while we
221 considered that DNA from *Plasmodium relictum* was detected in the corresponding
222 positive nestling. The only PCR-positive sample for *Plasmodium relictum* was also
223 positive for *Leucocytozoon* sp. (L-MILVUS1 haplotype) and we recorded three other
224 individuals with a co-occurrence of both *Leucocytozoon* haplotypes L-MILVUS1 and
225 L-MILANS4, among which two nestlings from the same brood. This suggests that
226 co-infections can occur relatively frequently, both for interspecific (1/47 individual)
227 and inter-haplotypes (3/47) co-occurrences.

228 At a same wing length, females are heavier than males (best LMER selected with
229 'Sex' as factor, $p < 0.0001$, Table 1. Estimate for males = -84.8, $df = 31.1$, $t = -5.45$).
230 Thus, the body condition of the nestlings is presented for both sex separately in Fig.
231 1. No difference of body condition was detected between infected and non-infected
232 kites whatever the sex ($p = 0.15$, Table 1).

233

234 **Discussion**

235 Our findings showed that nestlings of Red Kite are infected by haemosporidians,
236 notably *Leucocytozoon ssp.* and *P. relictum*, in three French breeding areas. The
237 prevalence of 28% we measured was higher than those commonly reported for
238 *Leucocytozoon* in Red Kite chicks from German, Spanish, UK or Danish populations
239 while *Plasmodium* was less frequently found (Table 2). Overall, if we aggregate all
240 the studies reported in Table 2 except our and without distinction of age, the
241 prevalences measured in the Red Kite (n=366) were 3.8%, 0.3% and 0.0% for
242 *Leucocytozoon*, *Haemoproteus* and *Plasmodium*, respectively. Co-infection by two
243 haplotypes of *Leucocytozoon* corresponding to some of those previously described
244 in Spanish populations of Red Kite (Pérez-Rodríguez et al. 2013) were found in
245 three nestlings of the French populations studied. We also point out that the
246 detection of *P. relictum* in one single nestling of a French population is the first
247 record for the Red Kite while several haplotypes of *Plasmodium* were detected in
248 the Black Kite *Milvus migrans* (Pérez-Rodríguez et al. 2013; Nourani et al. 2020).
249 More generally, *P. relictum* has already been reported in species belonging to
250 Accipitridae and Falconidae families (Bennett et al. 1993). It is considered as a
251 serious pathogen for birds and has been associated to virulent diseases and even
252 death for translocated Gyrfalcon *Falco rusticolus* (Remple 2004). However, to our
253 knowledge, no information is available on its harmfulness for Accipitridae and the
254 individual infected by *P. relictum* we found did not show obvious health troubles.

255 Prevalence of blood parasites in diurnal raptors is highly variable and may reach
256 50% or more notably in adults (e.g., Forrester et al. 1994; Castellucci et al. 1998),
257 the genera *Haemoproteus* and *Leucocytozoon* being the most frequently detected
258 while *Plasmodium* commonly has lower prevalence (Remple 2004). Overall,

259 nestlings have been reported to exhibit relatively low prevalence of Haemosporida in
260 numerous studies (e.g., Gutiérrez-López et al. 2015; Hanel et al. 2016) even if
261 notable exceptions exist (Toyne and Ashford, 1997, Jeffries et al. 2015). Such
262 differences of prevalences between juveniles and adults have been explained by the
263 higher probability of adults contracting an infection in successive year (Podmokła et
264 al. 2014). For the Red Kite, available data does not allow to identify a difference of
265 prevalence between adults and nestlings mainly because of the low number of
266 positive individuals and unbalanced samples (Table 2).

267 The comparison of Haemosporida prevalence of Red Kite in Spain to those
268 determined in other studies (Table 2) suggests that it could be higher in more
269 northern areas such as France and potentially in Denmark, UK and Western Europe
270 despite low sample sizes. The prevalence of haemosporidians in diurnal raptors has
271 been explained by different factors. Some are related to populations such as the
272 abundance of Diptera vectors within the environment (Sol et al. 2003, Sehgal 2015)
273 while others rely more on species features, such as the world geographic range,
274 breeding habitat and embryonic development (Tella et al. 1999). In the case of the
275 Red Kite, abundance of vectors has never been investigated directly in the previous
276 studies published but it was assumed that the low prevalence measured in Spain
277 could be due to the dryness of the environment, making it unsuitable for the main
278 vectors of haemosporidian parasites (Tella et al. 1999). In Franche-Comté, the
279 climate is more wet (annual rainfall from 1000 to 1500 mm against 600 mm in Spain)
280 and numerous ponds, lakes and streams composed the landscape of the studied
281 areas, which could at least partly explain the relatively high prevalence of
282 *Leucocytozoon*. Moreover, the world range of the Red Kite limited to Western
283 Europe, the absence of inter-continental migration notably through Africa and the
284 fact that it breeds in open-woodland habitat are all together factors that may explain

285 relatively low Haemosporida prevalence in raptors and more generally animals
286 (Tella et al. 1999; Altizer et al. 2011).

287 Lower body condition has been related to infection status (i.e., infected or not) by
288 haemosporidians in both passerines (Garvin et al. 2006, Hatchwell et al. 2000) and
289 raptors (Dawson and Bortolotti 2000). We did not show any effect of infection of Red
290 Kite by *Leucocytozoon* on body condition, which suggests that these blood parasites
291 might have no or weak pathogenic consequences on nestlings and/or that the
292 infection intensity is too low to impair condition. In the American kestrel *Falco*
293 *sparverius*, Dawson and Bortolotti (2000) found that a reduced condition of males
294 was associated with higher intensity of infections by *Haemoproteus* using blood
295 smear as a diagnostic tool. Moreover, Schoenle et al. (2017) reported that
296 measuring intensity infection rather than only presence/absence could be important
297 when evaluating the role of infection in influencing hosts' behaviour, physiology or
298 fitness. If the molecular methods we used are considered as relevant to diagnose
299 infection status of Red Kite by blood parasites (Martinsen et al. 2006, Krone et al.
300 2008), blood smears remain useful to measure the infection intensity in individuals
301 and should be implemented complementarily to PCR screening in further studies.

302 Overall, both the low to medium prevalences reported in the different European
303 populations studied and the absence of negative correlation with body condition we
304 show suggest that hematozoans in Red Kite nestlings could be a limited threat for
305 this life stage even if the detection of *P. relictum* in one chick questions about its
306 occurrence at a larger scale and possible health effects on some individuals. In
307 addition, recent findings showed that low-level chronic malaria infection reduced life
308 span as well as the lifetime number and quality of offspring in a wild population of
309 great reed warblers *Acrocephalus arundinaceus*, these delayed fitness effects of
310 malaria being mediated by telomere degradation (Asghar et al. 2015). This stresses
311 the need for further research on "subtle" hematozoan effect on individual in different

312 avian groups including raptors. Moreover, the prevalence in adults has been only
313 assessed in Spain, a country where it is particularly low, while breeder's survival is a
314 critical parameter in the dynamic of raptor populations (Saether and Bakke 2000).
315 Further investigation associating both qualitative and quantitative methods on a
316 significant number of Red Kites and including adults, from other European areas,
317 notably in Germany and France, would be needed to confirm whether blood
318 pathogens can be considered as negligible threat for the Red Kite population.

319

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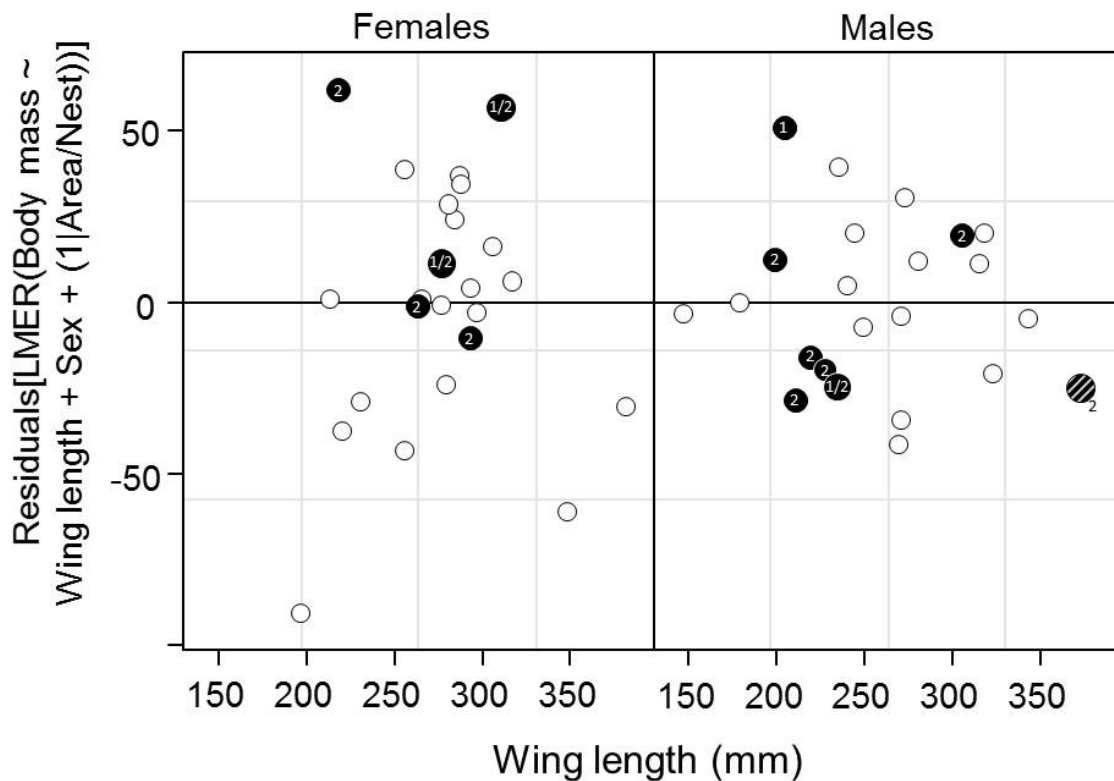
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433 *Figure caption*

434 **Fig. 1** Body mass of the 47 Red Kite chicks studied standardised against wing
 435 length taken as a proxy of age (Mougeot and Bretagnolle 2006) for females and
 436 males separately. The individuals for which DNA from *Leucocytozoon* ssp. was
 437 detected in blood were shown as a black circle in which the corresponding
 438 haplotype 1 (L-MILANS4, Genbank accession number JN164713) and/or 2 (L-
 439 MILVUS1, JN164716) is reported. The grey and black hatched dot corresponds to
 440 the male infected by both *Leucocytozoon* (haplotype 2) and *Plasmodium relictum*.



441

442 **Table 1** Linear mixed-effect models tested to explain body mass as response. The best
 443 model selected based on corrected Akaike information criteria is reported in bold (LL:
 444 maximized log-likelihood; K: number of estimated parameters; AICc: Corrected Akaike's
 445 information criterion; w_ic: Akaike weight)

Model tested (Body mass ~)	LL	K	AICc	Δ AICc	w_ic
Wing length + Sex + (1 Area/Nest)	-258.9	6	532.0	0.00	0.39
Wing length + Sex * Status + (1 Area/Nest)	-256.5	8	532.9	0.89	0.25
Wing length + Sex + Status + (1 Area/Nest)	-258.1	7	533.0	1.03	0.23
Wing length * Sex + (1 Area/Nest)	-258.7	7	534.2	2.20	0.13
Wing length + (1 Area/Nest)	-266.1	5	543.6	11.57	0.00

446

447 **Table 2** Summary of the data available on Red Kite infection by the haemosporidian
 448 parasites *Leucocytozoon spp.* (*Leuco.*), *Haemoproteus spp.* (*Haemop.*) and
 449 *Plasmodium spp.* (*Plasmo.*). (n = total number of screened individuals; *nr* = not
 450 reported; *ns* = not screened)

Area/country	Age	n	Prevalence in % [<i>n</i> of infected kites]			Reference
			<i>Leuco.</i>	<i>Haemop.</i>	<i>Plasmod.</i>	
Western Europe	<i>nr</i>	4	25 [1]	25 [1]	0 [0]	1
Germany	Nestling	13	15 [2]	0 [0]	0 [0]	2
	Juvenile	2	0 [0]	0 [0]	0 [0]	
	Adult	9	0 [0]	0 [0]	0 [0]	
	<i>nr</i>	2	0 [0]	0 [0]	0 [0]	3
UK	<i>nr</i>	1	0 [0]	0 [0]	0 [0]	4
Spain	<i>nr</i>	46	0 [0]	0 [0]	0 [0]	5
	Juvenile	164	4 [6]	0 [0]	0 [0]	6
	Adult	104	2 [2]	0 [0]	0 [0]	
Denmark	Nestling	17	18 [3]	0 [0]	0 [0]	7
	Juvenile	1	0 [0]	0 [0]	0 [0]	
	Adult	1	0 [0]	0 [0]	0 [0]	
France	Nestling	47	28 [13]	0 [0]	2 [1]	This study

451 1: Peirce (1981), 2: Krone *et al.* (2001), 3: Krone *et al.* (2008), 4: Peirce *et al.* (1983), 5: Tella *et al.* (1999),
 452 6: Pérez-Rodríguez *et al.* (2013), 7: Laursen (2008).