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Plasmodium spp. In a captive raptor collection of a safaripark in northwest Italy F.E. Scaglione a,*, F.T. Cannizzo a, L. Chiappino a, A. Sereno a, M. Ripepi b, S. Salamida c, E. Manuali c, E. Bollo a
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Blood parasites infect all vertebrates (Clayton and Moore 1997). Avian malaria parasites (*Plasmodium* spp., *Plasmodiidae*) are cosmopolitan in their distribution and are responsible for severe diseases in domestic and wild birds. In September 2009, nine raptorial birds that either arrived recently or were maintained as permanent residents at the Safaripark Pombia (northwest Italy) showed loss of stamina, developing listlessness, anorexia and regurgitation. Within one month three animals died and were necropsied. Following the diagnosis of *Plasmodium* infection all other raptorial birds were treated: clinical improvement was observed in all birds, and blood smears made after one month resulted negative for parasites.

Keywords:

Bird, Blood parasites, Captive raptor, Malaria, *Plasmodium* spp., Zoo

Blood parasites infect all vertebrates, and can affect the evolution and ecology of many species (Clayton and Moore, 1997). Infection of birds is common, and it is estimated that 68% of all bird species is susceptible to haemosporidians (Atkinson et al., 2000). Avian malaria parasites (*Plasmodium* spp., *Plasmodiidae*) are cosmopolitan in their distribution. They are relatives of human malaria parasites and are responsible for severe diseases in some domestic and wild birds, becoming the object of extensive investigation since the beginning of the 20th century (Valkiūnas et al., 2005). In field studies, several outbreaks of *Plasmodium* infections in wild birds are reported (Ricklefs et al., 2005; Sehgal et al., 2005; Bensch et al., 2009), but consequences of the infection in avian hosts are still poorly understood (Palinauskas et al., 2009). Avian haematozoa infection has been related to behavioural changes, sexual selection, and the reduction and extinction of native bird populations (Basto et al., 2006).

Zoological aviary gardens usually house and move birds with an unknown history of exposure to diseases. The parasites inevitably accumulate in the host bird and therefore may affect its ability to grow, survive, and reproduce; parasites can even alter behaviour, abundance, and distribution of an entire species (Varghese, 1987). Birds that are kept outdoors or in flocks are more likely to develop a parasites infection because of their increased risk of exposure.

Aim of our work is to describe a mortality event in raptors in an aviary garden in the northwest Italy due to haemoparasite infection.

In September 2009, nine raptorial birds that either arrived recently or were maintained as permanent residents at the Safaripark Pombia (northwest Italy) showed loss of stamina, developing listlessness, anorexia and regurgitation. Animals involved were: one Snowy Owl (*Bubo scandiacus*), two Barn Owls (*Tyto alba*), one Peregrine Falcon (*Falco peregrinus*), one Saker Falcon (*Falco cherrug*), one Saker Falcon $\frac{3}{4}$ (*Falco cherrug*), one hybrid falcon (Saker Falcon x Gyrsaker), one hybrid falcon (Gyrfalcon x Gyrsaker), and one Crested Caracara (*Caracara cheriway*). During the night the animals were kept in external or internal aviaries and during the day they were used for flight demonstrations. For cytological investigations blood samples were collected from the medial metatarsal vein, and smears were prepared, fixed with absolute methanol and stained with Giemsa stain. Slides were cover slipped and examined on a light microscope (LeicaDMLS2, Leica Microsystems, Wetzlar, Germany). For histological examination, tissue samples were fixed in 10% neutral buffered formalin (pH 7) and paraffin-embedded. Four- μ m sections were cut using a microtome (Leica Microsystems, Wetzlar, Germany), stained with haematoxylin and eosin, and examined by light microscopy (Leica DM LS2, Leica Microsystems, Wetzlar, Germany). Samples processed for ultrastructural evaluations were fixed in 2.5% gluteraldehyde phosphate (pH 7.3) and stored at 4 °C for 24 h. After the post-fixation process (in 1% osmium for 2 h and a quick wash out in 30% acetone) the samples were dehydrated in acetone and Spurr resin embedded. From each sample, using the ultramicrotome, thin sections (0.9 μ m) were obtained, stained with toluidine blue, and ultrathin sections of 70 nm contrasted by uranyl acetate and Pb citrate. The grids were evaluated using a transmission electron microscope (CM10 Philips, Koninklijke Philips Electronics N.V. 5621 BA, Eindhoven, The Netherlands). The genomic DNA was extracted from sampled organs (spleen, liver and lung) using a commercial DNA isolation kit (MACHEREY-NAGEL, Düren, Germany), according to the manufacturer's protocol. The extracted templates were used for the DNA amplification by means of a thermal cycler (Gene Amp PCR System2400, Perkin Elmer, Waltham, MA 02451, USA), using a specific primer set, as described (Hellgren et al., 2004). Briefly, a nested-PCR targeting the mitochondrial cytochrome b gene of *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. was performed. The first step of the PCR protocol was performed using HaemNFI [5'-CATATATTAAGAGAAITATGGAG-3'] and Haem NR3 [5'-ATAGAAAG ATAAGAAATACCATTC-3'] primers, to amplify parasite mtDNAs from species of *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. The reaction was performed in a final volume of 25 μ l, which included 2 μ l of the extracted DNA template, 1.5 μ M of each primer, and 12.5 μ l of HotStarTaq® Master Mix (Qiagen, Hilden, Germany). In the second step of PCR protocol HaemF (5'-TGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') primers (Bensch et al., 2000) for *Plasmodium* spp. and *Haemoproteus* spp., and HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-

CATTATCTGG ATGAGATAATGGIGC-3') for *Leucocytozoon* spp. were used. In the first step the DNA amplification was carried out, following a hot-start incubation (95 °C for 15min), under the following conditions: 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C for 20 cycles. A final elongation step was performed at 72 °C for 10min. In order to perform the second step, 4 µl of the first PCR product were used as template (2 µl for *Plasmodium* spp. and *Haemoproteus* spp. detection, and 2 µl for *Leucocytozoon* spp. detection, separately). The PCR conditions were the same as in the first step except for the number of cycles (35 cycles) (Scaglione et al., 2015). A positive DNA control for *Haemoproteus* spp., *Plasmodium* spp. or *Leucocytozoon* spp. was obtained from eagle owls (*Bubo bubo*) naturally infected as verified by blood smears examination and PCR (Ortego and Cordero, 2009). Each amplicon (480 bp) obtained from the second PCR step was visualized by means of agarose gel electrophoresis. All positive samples were sequenced by Macrogen Europe (Amsterdam, The Netherlands), with the primers used for the second step of the nested PCR. Sequences were manually edited using BioEdit 7.0.5.3 and cytochrome b gene haplotypes, defined by a sequence difference of at least one base in the amplified fragment, were identified using the Nucleotide BLAST application of GenBank (Pérez-Tris and Bensch, 2005). The Snowy Owl, who first presented the symptoms, had dyspnea, regurgitation and renal failure, and a *Trichomonas* infestation was diagnosed. Treatment with Baytril® (Bayer, Leverkusen, Germany) and Spartix® (Janssen, Beerse, Belgium) was initiated. No clinical improvement in the bird's condition became apparent and the animal died after five days. At necropsy, yellow plaques and raised cheesy masses in the mouth, pharynx, oesophagus, crop and proventriculus, due to *Trichomonas* spp., yellow to gray nodules or plaques in lungs, air sacs and kidneys due to *Aspergillus* spp. were observed. Histologically *Aspergillus* infection was confirmed and haemoparasites were observed in spleen and liver. At the same time one Saker Falcon and one Barn Owl showed anorexia and weakness. In the oral cavity and esophagus diffuse petechiae were observed. Intramuscular treatment with amoxicillin and clavulanic acid was initiated in the Saker Falcon, and within two weeks the Saker Falcon died. At necropsy the latter showed hepatic, lung and kidney hyperemia, and an ulcer in the tongue. Histological investigation revealed diffuse hepatic hyperemia, perivascular inflammatory foci and haemoparasites (Fig. 1a). In lungs a severe hyperemia, thickening of the intra-alveolar septa, presence of blood in the bronchi, and foci of necrosis associated with lymphocytic and heterophilic infiltrate was evident. Haemoparasites were also found in the myocardial tissue. The Barn Owl died after five days; necropsy revealed only hyperemia of meninges. Histologic findings were represented by a diffuse perivascular inflammation in the liver, diffuse hyperemia in lungs, heart and brain, spleen lymphocytes depletion; haemoparasites were observed in liver, spleen, lungs and heart (Fig. 1b). Transmission electron microscopy showed schizogony in endothelial cells of the lungs that were filled

with extraerythrocytic merozoites in intracytoplasmic large vacuoles that displaced the nucleus. Some parasites were also seen in extracellular space (Fig. 2). All organs tested by nested-PCR (spleen, liver and lung) resulted positive for *Haemoproteus/Plasmodium* spp. Samples that scored PCR-positive were sequenced but, despite repeated amplification, *Plasmodium* was detected only at genus level, probably due to a poor quality of the samples. Following the diagnosis of *Plasmodium* spp. infection by blood smears in all the remaining raptorial birds, all animals were treated with chloroquine (25 mg/kg b.w. followed after 12 h by 15 mg/kg b.w., after 24 h by 15 mg/kg b.w., and after a further 48 h by 15 mg/kg b.w.). Clinical improvement was observed in all birds, and blood smears obtained after one month resulted negative for haemoparasites. The effects of stress on the physiology and behaviour of birds as a consequence of their captivity, the proximity of different bird cages, the housing of more than one bird per cage, and a poor hygienic environment are important conditions responsible for the onset of disease (Varghese, 1987). Furthermore, zoological aviary gardens usually house and move birds for which the history of exposure to diseases is often unknown, increasing the risk of infection. It should also be taken into account the geographical location of the Safaripark Pombia, situated in an area surrounded by rice fields and close to an international airport. Both conditions may increase the presence of vectors and the diffusion of the disease. It is therefore important to emphasize the need for a careful sanitary control of newly introduced animals, to reduce the risk of spreading new diseases, and to adopt preventive measures to reduce the risk of multiple infections (Perez Cordon et al., 2009).

Although results of sequencing may appear incomplete, in association with clinical, histological, transmission electron microscopy and blood smears findings, they clearly indicate an outbreak of malaria in the safari park zoological collection.

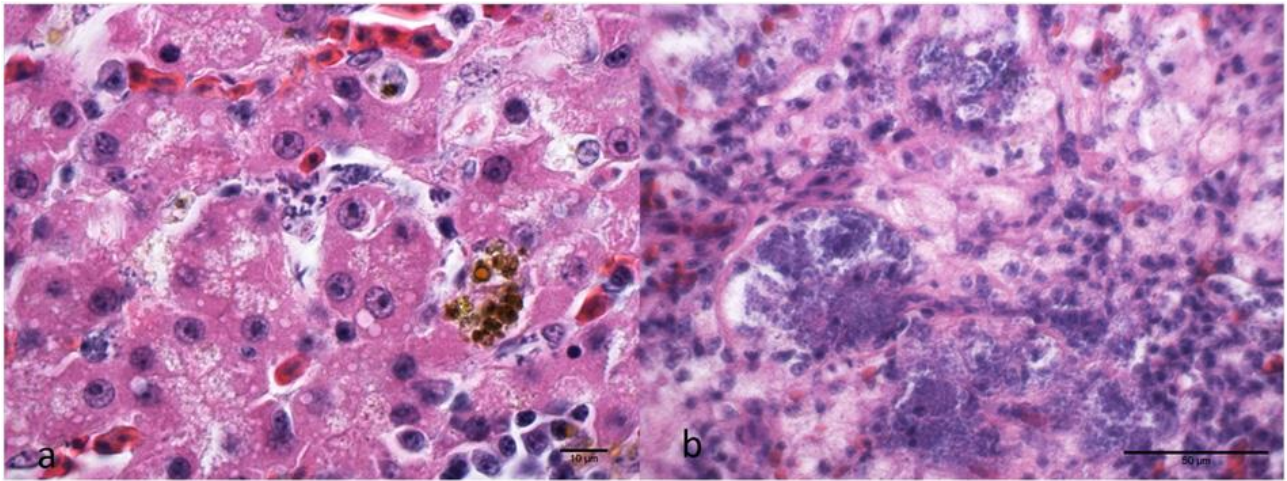


Fig. 1. a. Saker Falcon, liver: diffuse perivascular inflammation, hyperemia and Plasmodium parasites (HE); b. Barn Owl, spleen: lymphocytes depletion and Plasmodium parasites (HE)

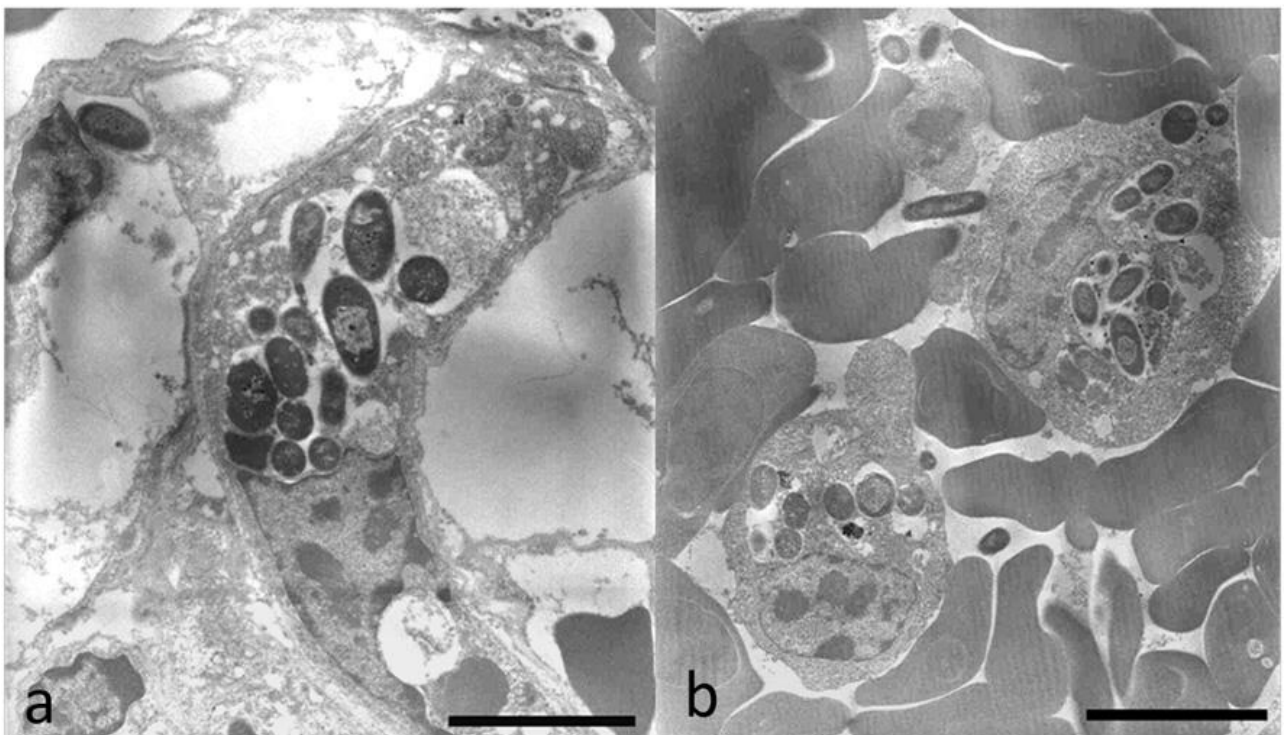


Fig. 2. a. Barn Owl, lung: endothelial cells and alveolar macrophage of the lungs with merozoites in intracytoplasmic large vacuoles displacing the nucleus (TEM bar 5 µm); b. Barn Owl, lung: large aggregates of merozoites in endothelial cells (TEM bar 5 µm).

Conflicts of interest None.

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