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Fatal avian malaria in captive Atlantic puffins (*Fratercula arctica*) in Switzerland

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ABSTRACT

Avian malaria is a vector-borne disease caused by *Plasmodium* species, which may affect a broad spectrum of bird families worldwide. In most endemic and migratory birds, *Plasmodium* infections seem not to cause severe harm; however, non-indigenous species kept in human care such as penguins may experience high morbidity and mortality rates. Fatal avian malaria may also occur in other non-native seabirds such as puffins (*Fratercula* spp.), but reported cases are scarce.

The aim of this study was to analyze seven cases of sudden death in captive Atlantic puffins (*Fratercula arctica*) at Berne Animal Park in Switzerland between 2010 and 2020, and to determine the involvement of haemosporidian parasites in the fatal outcome. In all cases, lymphoplasmacytic inflammation, necrotic lesions in several organs and presence of protozoan stages within tissues/erythrocytes or accumulation of iron-based pigment were observed histologically. A one-step multiplex PCR designed to simultaneously detect and discriminate haemosporidia belonging to the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, and a nested PCR detecting *Plasmodium* and *Haemoproteus* infections were performed on DNA extracted from formalin-fixed and paraffin-embedded (FFPE) or fresh liver and spleen tissues from five and two birds, respectively. *Plasmodium* spp. DNA was detected in the tissues from six of seven birds by the one-step multiplex PCR and in five of seven individuals by the nested PCR protocol. Direct sequencing of the amplification products allowed the molecular identification of *Plasmodium relictum* SGS1 as the involved species in three individuals and *Plasmodium matutinum* LINN1 in two of these fatal cases. In one bird, no haemosporidian DNA could be amplified from FFPE tissues despite of suggestive histopathological findings. These results indicate that avian malaria represents an important cause of death in captive puffins and it should be considered as a differential diagnosis in unclear or fatal cases in this threatened bird species.

1. Introduction

Avian malaria is a parasitic disease caused by ecologically-successful protozoa belonging to the genus *Plasmodium*, which infect a wide variety of bird species (Valkiūnas, 2005; Atkinson, 2008) and are transmitted by numerous mosquito species. The infection shows a worldwide distribution except for the Antarctica (Redig et al., 1993) and reaches high prevalence amongst endemic and migratory birds (Hayworth et al., 1987; Huijben et al., 2007). Although the parasite appears to cause little harm in the native avian population, it can have grave consequences for non-indigenous bird species, such as exotic birds kept in human care

(Huijben et al., 2007). Fatal *Plasmodium* infections were reported in different penguin species (Family Spheniscidae), causing high mortality rates in untreated juveniles and previously unexposed adults from several zoos around the world (Grilo et al., 2016; Vanstreels et al., 2016). Since penguins are extremely popular seabirds kept in zoos worldwide, it is not surprising that most fatal cases of avian malaria were described in members of the family Spheniscidae. However, the lacking history of co-evolution between the parasite and marine bird species suggest that seabirds in general may be highly susceptible to avian malaria. Cases of clinical avian malaria have been also scarcely observed in Atlantic puffins (*Fratercula arctica*) kept in captivity (Loupal

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and Kutzer, 1996). Atlantic puffins are marine birds belonging to the family Alcidae and can be found along rocky shores and cliffs throughout the North Atlantic and Western Arctic Oceans (BirdLife International, 2020). Being highly susceptible to the impacts of climate change and marine pollution, this species experienced rapid declines across most of its European range. As a consequence, the Atlantic puffin has been classified as vulnerable by the IUCN since 2015 and is therefore considered as globally threatened (BirdLife International, 2020). Nevertheless, the species is only sparsely observed in human care. Among Species 360 ZIMS (Zoological Information Management Software: <https://www.species360.org>) affiliated institutions, puffins are only kept in eight zoos in Europe, five zoos in North America, one zoo in Singapore and one institution in Japan with a global total number of 164 individuals (accessed December 2020). In Switzerland, the species is exclusively held at Berne Animal Park (December 2020: 13 animals), where avian malaria was assumed to be the second most common cause of death after aspergillosis (Rosset and Blatter, 2019).

A broad spectrum of about 55 avian *Plasmodium* morphospecies can be distinguished based on morphological features of blood stages and DNA sequence information (Valkiūnas and Iezhova, 2018). Within the *Plasmodium* morphospecies, hundreds of genetic lineages have been identified based on comparison of the cytochrome *b* gene (*cyt-b*) suggesting the existence of significant intra-species variation, especially for *P. relictum* which may in fact be designated as species complex (Bensch et al., 2009; Valkiūnas et al., 2018). *Plasmodium relictum* was the first described and most frequent agent of avian malaria (Grassi and Feletti, 1891), which may be associated with its ability to infect numerous mosquito species as well as a wide range of birds (Valkiūnas, 2005; Huijben et al., 2007). It is able to infect about 300 different bird species belonging to 11 orders (Valkiūnas, 2005; Hellgren et al., 2015).

Transmission of *Plasmodium* species affecting birds occurs through the bite of blood-feeding diptera belonging to the genera *Aedemoyia*, *Aedes*, *Anopheles*, *Armigeres*, *Coquillettidia*, *Culex*, *Culiseta*, *Mansonia*, *Psorophora* and *Wyeomyia* (Valkiūnas, 2005). *Culex* spp. appears to be the most significant and most successful vector (Kimura et al., 2010; Glazot et al., 2012). Although pursuit-diving seabirds are protected by their dense plumage to a certain extent, the vectors find exposed skin to feed in the areas surrounding the eyes, legs, feet and beak (Grilo et al., 2016).

The development of the parasite within the dipteran vector (sexual reproduction) depends on weather conditions and takes approximately seven days during summer (Huijben et al., 2007). After invading the vertebrate host's blood stream, the parasite undergoes an asexual phase including exoerythrocytic and erythrocytic cycles multiplying as haploid clones. The incubation time from inoculation of sporozoites by female mosquitoes until development of clinical signs takes up to five days for *P. relictum* (Valkiūnas, 2005). If clinical signs occur in the avian host, they are not pathognomonic (Valkiūnas, 2005; Atkinson, 2008). Due to the fact that avian *Plasmodium* spp. and their hosts have co-evolved since ancient times in Europe, various bird species do not show any clinical signs during infection (Bennett et al., 1993a; Huijben et al., 2007). However, avian populations, which had been geographically isolated from the parasite and its vectors, can be highly susceptible to the disease (Warner, 1968; Beier and Stoskopf, 1980; Huijben et al., 2007; Dinhopf et al., 2011; Wallace, 2014). Depressed demeanor, lethargy, inappetence up to anorexia followed by weight loss, fluffed or ruffled plumage, regurgitation, vomiting, pale mucous membranes and respiratory distress subsequently occur in naïve individuals (Rodhain, 1939; Griner and Sheridan, 1967; Fleischman et al., 1968; Stoskopf and Beier, 1979; Hayworth et al., 1987; Fix et al., 1988; Loupal and Kutzer, 1996; Grim et al., 2003; Bueno et al., 2010). Having not yet acquired a complete immune response, juveniles are considered to be at highest risk (Beier and Stoskopf, 1980; Sol et al., 2003; Huijben et al., 2007; Dinhopf et al., 2011; Knowles et al., 2011; Wallace, 2014). Sudden cases of death may occur after an extremely short period of acute clinical signs, but also without any change in the animal's general condition (Stoskopf and

Beier, 1979; Loupal and Kutzer, 1996). The prognosis for individuals with clinical signs is usually poor (Graczyk et al., 1994b). Consequently, rapid diagnosis of the disease is essential. In most zoos and animal parks keeping penguins or puffins in outdoor enclosures, preventive measures including chemoprophylaxis throughout the mosquito season are taken (Huijben et al., 2007; Cereghetti, 2012).

Different studies encourage using a combination of microscopic examination and molecular methods for the analysis of avian haemosporidian parasites (Valkiūnas et al., 2006, 2008a). Microscopy, providing an inexpensive and generally available method in any laboratory, is fundamental for determination and verification of an infection's identity and intensity (Valkiūnas et al., 2008a). Despite these advantages, it must be noted that the sensitivity clearly depends on the blood smears' quality and the investigators' skills with its main shortcoming of low sensitivity when it comes to light parasitemia, which can be easily overlooked by this method (Valkiūnas et al., 2008a). Also, distinction between *Plasmodium* and other avian haemosporidia like *Haemoproteus* may be difficult (Fallon and Ricklefs, 2008) for inexperienced investigators. Nevertheless, regarding mixed infections with haemosporidians belonging to the same or different genera and subgenera, which are very common in some bird species (Valkiūnas et al., 2003), microscopy is more reliable than PCR analyses (Valkiūnas et al., 2006).

In the present study, cases of sudden death in three male and four female Atlantic puffins (*Fratercula arctica*) at Berne Animal Park between 2010 and 2020 were investigated. In all cases, an infection with haemosporidian parasites was suspected either due to the clinical signs or based on the histopathological findings. The aim of the study was to confirm this assumption and to determine the involved haemosporidian species in the fatal cases. Due to the fact that Atlantic puffins are marine birds and non-native in Switzerland, we hypothesized that avian malaria presents an important differential diagnosis in cases of sudden death in this avian species.

2. Materials and methods

2.1. Study facility

Due to the threat of avian malaria, several preventive measures are important for the husbandry of susceptible bird species. At the Animal Park Berne, stagnant water is strictly avoided within the Puffin Pool with a wave machine and current pumps to prevent the mosquitoes' development. Air conditioning is maintained by a ventilation system, producing a wind speed of approximately 8 m per second 50 cm above the water surface. This ensures a constant breeze across the water surface to keep the air temperature at a low level, but also to make the Puffin Hall unattractive for mosquitoes (Rosset and Blatter, 2019). Furthermore, the Puffin Hall is aligned against the sun in a way that a cold reservoir is formed in the area of the enclosure. As a consequence, the water temperature does not exceed 12 °C, not even in midsummer. Additionally, food additives ("Anti-Malaria" and "Anti-Aspergillosis": Tiere spezial, Wasservogel Island, Papageitaucher POC and HEP, Plocher® integral-technik, Zeller Umweltsysteme, D-29339 Wathlingen) with a similar effect mechanism as homeopathic remedies, are mixed with the puffins' pellets (Lundi Ibis-See- (Eis-) Ente-Spezial, Lundi Deutschland, D-33415 Verl) (Rosset and Blatter, 2019).

2.2. Cases

In this study, cases of sudden death in three male and four female Atlantic puffins from Berne Animal Park between 2010 and 2020 were analyzed (Table 1).

2.3. Blood smears

From the individual exhibiting a short episode of acute clinical signs

Table 1
Cases of sudden death in Atlantic puffins from Berne Animal Park between 2010 and 2020.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Number of puffins^a	22	20	26	19	16	14	13
Birth date	June 01, 2009	July 17, 2012	June 27, 2014	June 21, 2015	July 19, 2018	August 01, 2008	August 01, 2008
Death date^b	August 13, 2010	July 31, 2013	August 17, 2015	August 17, 2018	September 12, 2019	September 04, 2020	September 14, 2020
Age at death	1y 2 mt 12d	1y 14d	1y 1 mt 21d	3y 1 mt 27d	1y 1 mt 24d	12y 1 mt 3d	12y 1 mt 13d
Sex	Female	Male	Female	Male	Female	Female	Male
Anamnesis	Found dead, no prior clinical signs observed	Found dead, no prior clinical signs observed	Found dead, no prior clinical signs observed	Found dead, no prior clinical signs observed	General condition moderately reduced, episode of dyspnea including beak breathing after handling, ventro-dorsal and latero-lateral radiographs within normal limits, severe infestation with <i>Plasmodium</i> sp. identified by modified Wright's stained peripheral blood smears	Found dead, no prior clinical signs observed	Found dead, no prior clinical signs observed
Therapy	N/A	N/A	N/A	N/A	Quarantine, force-feeding with fish and additional Critical Care® for carnivores (Oxbow PetProducts, USA), single dose of Amynin® subcutaneously, combination of atovaquone and proguanil (Malarone junior®) perorally	N/A	N/A

N/A: not applicable.

^a Number of puffins at Berne Animal Park on the date of death of each case.

^b Summer in the Northern hemisphere (Switzerland).

before death, consisting of moderately reduced general condition and an episode of dyspnea including beak breathing after handling (case 5), whole blood was collected ante mortem. Blood smears were performed following standard protocol with subsequent staining using the modified Wright's method at the Veterinary Laboratory at the University of Zurich (Fig. 3).

2.4. Necropsy and histopathological examination

Gross necropsy as well as histopathological examination were performed on all seven puffins. Various organs were sampled at necropsy and fixed in 4% buffered formalin for 24 h in the cases that were dissected at the Institute of Animal Pathology at the University of Bern (case 3 and 5–7). The necropsy of the other cases (case 1, 2 and 4) was performed at Berne Animal Park and formalin-fixed samples of different organs were sent to the Institute of Animal Pathology. Therefore, these tissue samples were fixed in formalin for longer than 24 h. All samples were then processed for routine histopathological examination: i.e. formalin-fixed tissues were embedded in paraffin, cut at 4 µm, mounted on glass slides and stained with hematoxylin and eosin (H&E).

2.5. DNA extraction

DNA was extracted from 20 µm-thick formalin-fixed and paraffin-embedded (FFPE) sections of liver and spleen (case 1–5) as previously described (Müller et al., 2003) or from 25 mg fresh liver tissue and 10 mg spleen tissue (case 6 and 7) using the DNeasy Blood & Tissue Kit (QIAGEN: Spin-Column Protocol for Animal Tissues) according to the manufacturer's protocol.

2.6. One-step multiplex PCR

A one-step multiplex PCR for simultaneous detection and discrimination of protozoa belonging to the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, targeting mitochondrial genome sequences was performed as previously described (Ciloglu et al., 2019). Three primer sets (Microsynth AG, Balgach, Switzerland) were used at a 10 µM working solution in a single reaction tube. Per tube, 0.2 µl of each primer (PMF, PMR, HMF, HMR, LMF, LMR) were mixed with 5 µl of a commercial master mix (QIAGEN Multiplex PCR Kit, Hombrechtikon, Switzerland), 1.8 µl of ddH₂O and 2 µl of DNA template to a total volume

of 10 µl. The PCR amplification was performed under the following conditions: 95 °C for 15' (for activation of HotStarTaq DNA Polymerase) followed by 35 cycles of denaturation at 94 °C for 30", annealing at 59 °C for 90" and extension at 72 °C for 30", followed by a final extension at 72 °C for 10'. Amplification products were resolved by gel electrophoresis in 2% agarose gels containing ethidium bromide for 1 h at 90 V. Three known positive DNA controls (*Plasmodium matutinum* LINN1 from *Spheniscus demersus*, *Haemoproteus* sp. from *Strix nebulosa*, *Leucocytozoon* sp. from *Parus major*) and one negative control (ddH₂O) were used for each run. In cases, in which the obtained sequences were identical to the used positive control, the DNA extraction from new FFPE sections was performed in a different laboratory and the PCR was repeated using DNA of another *Plasmodium* species as positive control in order to exclude the possibility of an eventual cross-contamination.

PCR products showing the expected band sizes were excised from the agarose gel, purified using the Zymo Research purification kit (Zymo-clean™ Gel DNA Recovery Kit, Zymo Research Europe GmbH, Lucerne, Switzerland) and sequenced in both directions with the same primers used in the PCR reactions (Microsynth AG, Balgach, Switzerland). The obtained sequences were then compared to those available on GenBank using the Nucleotide BLAST algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.7. Nested PCR

Research on avian haemosporidia has coalesced around the usage of the curated avian malaria database MalAvi, which consolidates all the information on cytochrome b (cyt-b) sequences for these protozoa (Valkiūnas et al., 2008b; Bensch et al., 2009). Each unique cyt-b sequence receives a lineage name (>1500 unique haemosporidian lineages are currently available in the database) and can be compared with newly obtained sequences using the BLAST function. Consequently, a nested PCR protocol detecting *Plasmodium* and *Haemoproteus* infections targeting the cyt-b gene was performed as previously described (Waldenström et al., 2004). An external and an internal primer set (Microsynth AG, Balgach, Switzerland) were used at a 100 µM working solution. In the initial reaction, each tube contained a total volume of 25 µl consisting of 0.15 µl of both primers from the external primer set (HAEMNF and HAEMNR2), 12.5 µl of a commercial master mix (QIAGEN Multiplex PCR Kit, Hombrechtikon, Switzerland), 9.7 µl of ddH₂O and 2.5 µl of DNA template. The initial PCR amplification was performed

under the following conditions: 95 °C for 15' (for activation of Hot-StarTaq DNA Polymerase), followed by 20 cycles of denaturation at 94 °C for 30", annealing at 50 °C for 90" and extension at 72 °C for 45" with a final extension at 72 °C for 10'. For the final PCR, 1.0 µl of the PCR products from the initial reaction was mixed with 0.15 µl of each primer from the internal primer set (HAEMF and HAEMR2) and the same reagents as in the initial PCR. The thermal profile was also identical, except for the number of cycles being increased from 20 to 35 cycles in the final reaction. Amplification products were resolved by gel electrophoresis in 2% agarose gels containing ethidium bromide for 35 min at 120 V. DNA was recovered from the PCR products using the Zymo Research purification kit (DNA Clean & Concentrator™-5, Zymo Research Europe GmbH, Lucerne, Switzerland). Bidirectional sequencing using the primers from the final reaction (internal primer set) was performed (Microsynth AG, Balgach, Switzerland), followed by comparison with sequences on GenBank using the Nucleotide BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and on the MalAvi database (<http://130.235.244.92/Malavi/blast.html>).

3. Results

The main macroscopical, histopathological and molecular findings of case 1 to 7 are summarized in Table 2

3.1. Case 1

At the postmortem examination, the liver and spleen were moderately enlarged. Histopathologically, the liver presented multifocal to coalescing, severe, subacute, periportal and perivascular lymphoplasmacytic infiltration. Round protozoan schizonts of up to 20 µm in diameter (Fig. 1A), which resembled haemosporidian stages, were associated with the lesions. Golden-brown pigment, birefringent under polarized light (accumulation of iron-based pigment) and phagocytized cell debris were frequently observed in Kupffer cells. The spleen presented a moderate, multifocal lymphohistiocytic inflammation, but no parasites were visible in this organ. The heart, lung and kidney were histologically unremarkable.

3.2. Case 2

At necropsy, the spleen was moderately enlarged, and the digestive tract was empty. At the histopathological examination, numerous intracytoplasmic haemosporidian schizonts of up to 10 µm in diameter, which contained numerous merozoites of 1–2 µm in diameter, were observed in endothelial cells of the liver (Figs. 1B and 2A), kidney, spleen, heart, lung, brain, proventriculus, ventriculus and caecum. Protozoan stages were also observed within few erythrocytes in some organs. The main histopathological changes involved liver and caecum, causing a multifocal, lymphocytic, portal hepatitis and diffuse, chronic,

lymphoplasmacytic typhlitis. The ileum, duodenum and pancreas were histologically unremarkable.

3.3. Case 3

At the postmortem examination, the mucosal membranes were pale, and the spleen and the liver were severely enlarged. The gastrointestinal tract was filled with physiological content, suggesting normal feeding behavior until death. At the histopathological examination, some erythrocytes in blood vessels of the liver, spleen, lung and kidney contained intracytoplasmic protozoal structures of 1–2 µm in diameter, consistent with haemosporidian stages. An infection with *Haemoproteus* was first suspected. In the liver, multifocal, periportal to diffuse, chronic, lymphohistiocytic and necrotizing inflammation with hepatocellular regeneration was observed, and numerous Kupffer cells contained iron-based pigment (Fig. 1C). In the spleen, severe, multifocal, follicular hyperplasia, and iron-based pigment accumulation in sinusoidal macrophages was observed. In the lung, a mild, multifocal, chronic, lymphohistiocytic, interstitial pneumonia was detectable, and some lung macrophages contained iron-based pigment. Additionally, multifocal lymphohistiocytic infiltrates in the submucosa and tunica muscularis of the glandular stomach and small intestine were present, and the kidney showed degeneration and necrosis of tubular epithelial cells with multifocal presence of iron-based pigment. The trachea, esophagus, heart, bursa of Fabricius and cloaca were histologically unremarkable.

3.4. Case 4

At necropsy, the spleen was severely enlarged, the gastrointestinal tract was only scarcely filled and pericardial effusion (serous fluid) was observed.

Histologically, numerous round to longish (15–30 µm) protozoal stages containing multiple basophilic, round, up to 1 µm in diameter big structures compatible with haemosporidian merozoites (Fig. 2B) were detectable in the blood vessels of the liver, the heart, the lung and the spleen. The heart, proventriculus, gizzard, small and large intestines and the brain were histologically unremarkable.

3.5. Case 5

Macroscopically, the animal was severely emaciated, the pectoral musculature was atrophied, and the liver was moderately enlarged. The gastrointestinal tract had scarce content. A white to gray, fuzzy membrane focally covered the cranial left air sacs. At the histopathological examination, this membrane consisted of fibrin exudate admixed with large numbers of 5–8 µm thick, branching, septated, parallel-walled fungal hyphae which occasionally formed conidiophores, compatible with *Aspergillus* sp., surrounded by degenerated heterophils and fewer macrophages. This inflammatory process extended deep into the

Table 2
Main macroscopical, histopathological and molecular findings of case 1 to 7.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Body weight (g)	370	Unknown	344	476	269	438	409
Body condition	Good	Good	Good	Good	Emaciated	Slightly reduced	Slightly reduced
Splenomegaly	+	+	+	+	+	+	+
Hepatomegaly	+	–	+	–	+	–	+
Distribution of tissue meronts	Liver	Liver, spleen, kidney, heart, lung, brain, proventriculus, ventriculus, caecum	Liver, spleen, kidney, lung	Liver, spleen, heart, Lung	No parasites observed	Liver, spleen	Liver, spleen, kidney, heart, lung, small intestine
One-step multiplex PCR result	<i>Plasmodium</i> sp.	<i>Plasmodium</i> sp.	<i>Plasmodium</i> sp.	Negative	<i>Plasmodium</i> sp.	<i>Plasmodium</i> sp.	<i>Plasmodium</i> sp.
Nested PCR result	<i>Plasmodium</i> sp.	Negative	<i>Plasmodium</i> sp.	Negative	<i>Plasmodium</i> sp.	<i>Plasmodium</i> sp.	<i>Plasmodium</i> sp.
Sequencing result	<i>Plasmodium relictum</i> SGS1	N/A	<i>Plasmodium relictum</i> SGS1	N/A	<i>Plasmodium relictum</i> SGS1	<i>Plasmodium matutinum</i> LINN1	<i>Plasmodium matutinum</i> LINN1

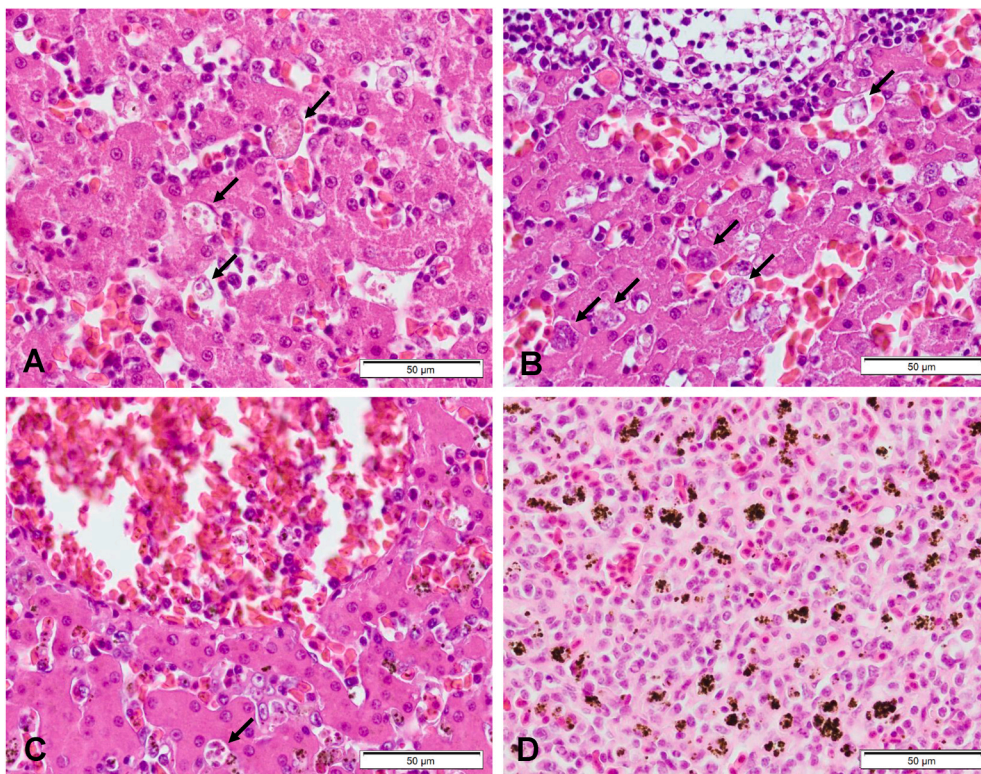


Fig. 1. H&E-stained histological sections of the liver (A, B, C) and the spleen (D) of captive Atlantic puffins at 400x magnification. A: Case 1, multiple protozoan *Plasmodium* schizonts of up to 20 µm in diameter (arrows). B: Case 2, periportal infiltration with lymphocytes and presence of multiple intracytoplasmic *Plasmodium* schizonts, which contain numerous merozoites (arrows). C: Case 3, *Plasmodium* merozoites within the liver parenchyma (arrow). D: Case 5, multiple histiocytes with intracytoplasmic brown, finely granular pigment (accumulation of iron-based pigment). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

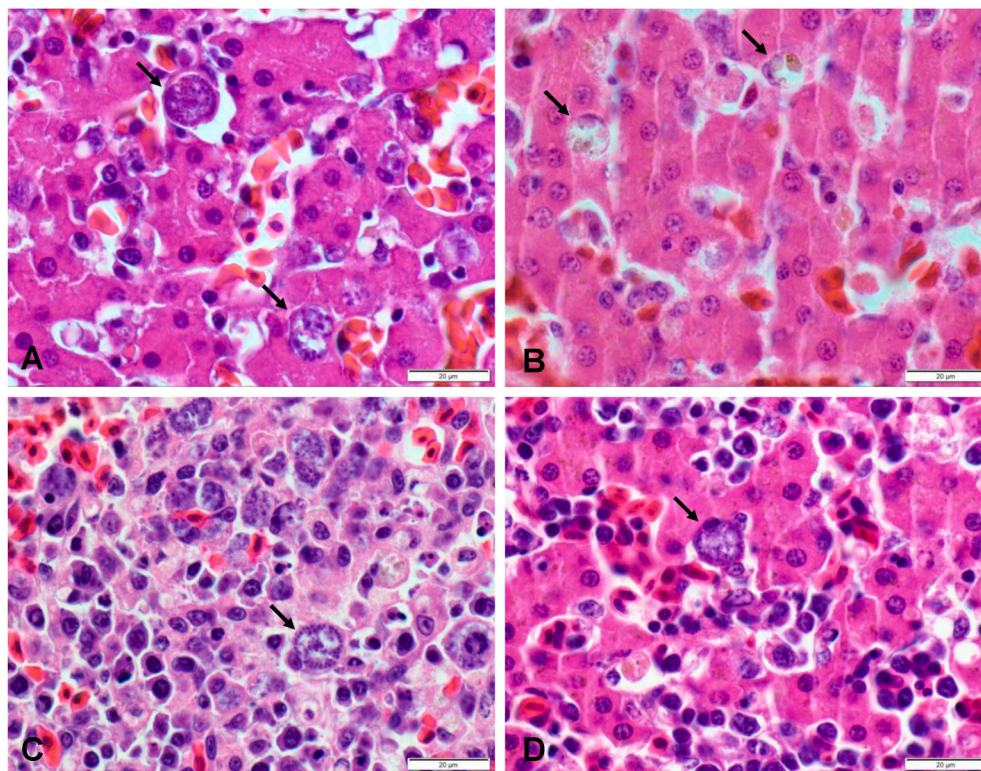


Fig. 2. H&E-stained histological sections of the liver (A, B, D) and the spleen (C) of captive Atlantic puffins at 1000x magnification, showing *Plasmodium* schizonts of up to 15 µm in diameter, containing multiple merozoites of 1–2 µm (arrows): A: Case 2, B: Case 4, C: Case 6, D: Case 7.

underlying tissue with severe, multifocal to coalescing necrosis of the skeletal muscle fibers. Similar inflammatory lesions and fungal hyphae were present in the pericardium and parabronchi. A histopathological

diagnosis of focal, severe, subacute, heterophilic and fibrinonecrotizing aerosacculitis and multifocal, moderate, subacute, heterophilic and fibrinous pericarditis and bronchiointerstitial pneumonia with fungal

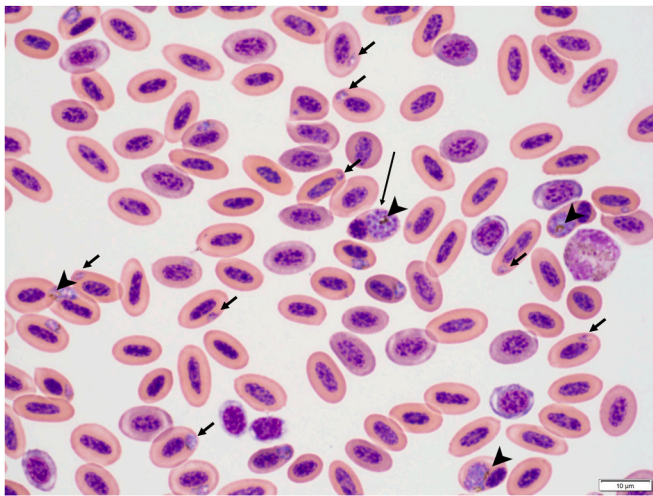


Fig. 3. Modified Wright-stained peripheral blood smear of a captive Atlantic puffin (case 5). Intra-erythrocytic stages of *Plasmodium relictum*: trophozoites (short arrows); mature meront (long arrow) with marked displacement of the erythrocyte's nucleus; pigment granules (arrowheads). Picture: Veterinary Laboratory, Vetsuisse Faculty, University of Zurich.

hyphae was made. Additionally, a multifocal, mild, periportal, chronic, lymphoplasmacytic hepatitis and a moderate, multifocal to coalescing, chronic histiocytic splenitis were observed. No structures suggestive of haemosporidian parasites were identified in the tissue sections, but iron-based pigment granules were present in the Kupffer cells in the liver and in the histiocytes of the spleen (Fig. 1D). The ventriculus, proventriculus and intestine were histologically unremarkable. Furthermore, intra-erythrocytic stages resembling *Plasmodium* sp. were observed in modified Wright-stained blood smears taken during the short acute phase of clinical signs (Fig. 3).

3.6. Case 6

At necropsy, the liver showed multifocal, up to 1 mm in diameter big, well demarcated, red spots and the spleen was diffusely severely enlarged. Within the parenchyma of the kidney, multifocal, whitish, moderately well demarcated, coarse nodules were visible.

Histologically, multifocal, intracytoplasmic basophilic microgranular organisms (compatible with *Plasmodium* merozoites) were detectable in the liver and the spleen (Fig. 2C) and some Kupffer cells contained brown-yellow, granular, intracytoplasmic iron-based pigment. Based on the histopathological examination, a multifocal, severe, subacute, lymphohistiocytic hepatitis with intralesional protozoa and a multifocal, severe, histiocytic splenitis with intralesional protozoal inclusions were diagnosed. The lung, heart, proventriculus, gizzard, intestine and kidney were histologically unremarkable.

3.7. Case 7

Macroscopically, a minimal bilateral atrophy of the pectoral muscles and a diffusely, severely enlarged liver were found. The main finding of the post-mortem examination was the lymphohistiocytic inflammation of several organs with intralesional protozoan cysts, severely affecting the spleen; moderately affecting the liver (Fig. 2D) leading to cholestasis, the myocardium, the interstitium of the kidney and mildly affecting the lung.

3.8. One-step multiplex PCR

In six of seven cases, a *Plasmodium* sp. infection could be confirmed by the one-step multiplex PCR on FFPE tissue samples (case 1–3 and 5)

or on fresh liver and spleen tissue (case 6 and 7), respectively. The method revealed negative results in case 4 (DNA extracted from FFPE tissue sections). In case 1 (MT568857), 3 (MT568859) and 5 (MT568860), the obtained sequences were identical to each other and shared a 100% BLAST identity (345/345 bp) with GenBank sequences of *Plasmodium relictum* from different avian species worldwide, such as *Spheniscus demersus* from South Africa (KY653774), *Passer domesticus* and *Loxia curvirostra* from Lithuania (KY653772, KY653773), and *Bubo scandiacus* and *Hemignathus virens* from the USA (KY653754, AY733090) (Beadell and Fleischer, 2005; Pacheco et al., 2018). The DNA sequence obtained in case 2 (MT568858), 6 (MW168984) and 7 (MW168985) showed a percent identity of 99.71% (344/345 bp) with GenBank sequences of *Plasmodium juxtanucleare* from *Myiarchus ferox* from Brazil (MG598392) (Ferreira-Junior et al., 2018), *P. lutzi* from *Diglossa lafresnayii* and *Turdus fuscater* from Colombia (KY653815, KC138226) (Mantilla et al., 2013; Pacheco et al., 2018), *P. elongatum* from *Spheniscus demersus* from South Africa (KY653802) (Pacheco et al., 2018) and *S. demersus* from USA (AY733088) as well as *P. relictum* from *Zenaida macroura* from the USA (AY733089) (Beadell and Fleischer, 2005; Valkiūnas et al., 2008b).

3.9. Nested PCR

An infection with *Plasmodium* sp. could be confirmed in five of seven cases by the nested PCR protocol using FFPE tissue samples in case 1, 3 and 5 and a pool of fresh liver and spleen tissue in case 6 and 7. The nested PCR from DNA, extracted from FFPE tissue sections, gave negative results in case 2 and 4. Comparison of the obtained sequences with those on GenBank and the *cyt-b* sequences on MalAvi revealed *Plasmodium relictum* SGS1 in case 1 (MW169032), 3 (MW169033) and 5 (MW169034) and *Plasmodium matutinum* LINN1 in case 6 (MW169036) and 7 (MW169037) with 100% BLAST identity (478/478 bp). According to MalAvi records, *P. relictum* SGS1 has already been reported in a wide variety of different avian orders (Anseriformes, Galliformes, Gruiformes, Charadriiformes, Ciconiiformes, Columbiformes, Passeriformes, Procellariiformes, Sphenisciformes, Strigiformes, Trochiliformes) in Africa, Asia, Europe, North and South America and Oceania. Also, *P. matutinum* lineage LINN1 is of cosmopolitan distribution being observed in Apterygiformes, Charadriiformes, Gruiformes, Passeriformes, Sphenisciformes and Strigiformes in Asia, Europe, North America and Oceania.

4. Discussion

Avian Malaria is a well-known health issue in different penguin species held in outdoor zoological exhibits due to the potential contact with arthropod vectors that may transmit *Plasmodium* spp. Even though non-indigenous bird species are generally thought to be severely harmed by avian haemosporidia, there are only very few records about *Plasmodium* sp. infections in puffins (Loupal and Kutzer, 1996). Our study provided additional evidence supporting the fact that this marine bird species is highly susceptible to avian malaria with fatal outcome. Although avian malaria was not detected in wild populations of any Alcidae species thus far (Quillfeldt et al., 2011), there is a wealth of evidence showing that global warming may create new favorable ecosystems for *Plasmodium* transmission in northern latitudes (Garamszegi, 2011); suggesting that also the risk of infection for puffins in their natural habitat might be increasing.

Native wild avian species, particularly passerines, are known to be frequently infected with *Plasmodium* spp. (Bennett et al., 1993b; Dufva, 1996; Quillfeldt et al., 2011; Valkiūnas et al., 2017). Since passerine birds definitively live in close proximity and even within Berne Animal Park, it is possible that local avifauna acted as a source of infection for the puffins, similarly to what has been speculated to occur in captive penguins (Beier and Trpis, 1981; Bueno et al., 2010; Dinhopf et al., 2011, 2015). Some studies detected identical *Plasmodium* spp. sequences

in native bird species and captive penguins (Bueno et al., 2010; Dinhopf et al., 2015), suggesting that *Plasmodium* spp. lineages infecting captive birds will reflect those present in the regional avifauna (Vanstreels et al., 2015). In neighboring countries of Switzerland, vector-transmitted blood parasites are abundant in several bird families. In Southwest Germany, prevalence of haemosporidian parasites is reported to be high in carrion crows (*Corvus corone*) with a prevalence of 29.5% for *Plasmodium* spp. (Schmid et al., 2017).

Besides, also other species of captive birds in the Animal Park could have been involved as infection source for the puffins. Amplified *Plasmodium* DNA sequences from mosquitoes, captured in a zoo in Japan, were identical to those of avian *Plasmodium* lineages previously detected in captive birds within that zoological garden (Ejiri et al., 2009). However, since no screening study for avian malaria was performed at Berne Animal Park, this is so far only a hypothesis.

The overall prevalence of avian *Plasmodium* DNA found in examined mosquito pools in Eastern Austria was 6.43% (Schoener et al., 2017). This prevalence is comparable to studies of *Culex pipiens* in Switzerland, that found prevalences of 6.6% in 2006–2007 (Glaizot et al., 2012) and 13.1–20.3% in 2010 and 2011 (Lalubin et al., 2013), respectively. In France, a prevalence of 0% (February) to 15.8% (October) was observed (Zélé et al., 2014) when examining the presence of haemosporidia in *Culex pipiens*.

The individuals in this study died within a time span between the end of July and mid-September (summer in the Northern hemisphere), that corresponds with observations in other studies reporting high mortality in penguins (Graczyk et al., 1994b; Huijben et al., 2007; Chitty et al., 2015) and puffins (Loupal and Kutzer, 1996) during summer or early autumn, with most cases occurring between June to October. Another study associated the peak in mortality in the third week of August with the maximum in larval mosquito densities two weeks before (Huijben et al., 2007). This coincides with a study from Austria reporting the highest number of avian *Plasmodium* positive mosquitoes in August (Schoener et al., 2017).

Four of the seven puffins were between 12 and 15 months old and compared to reported cases in Austria older at the time of death (Loupal and Kutzer, 1996). Consequently, these individuals survived the first mosquito season and died during the next year's vector season. We therefore suspect, that the chicks may have benefitted from maternal antibody protection, since in penguins specific anti-*Plasmodium* spp. immunoglobulins are transmitted through the egg yolk. These maternal antibodies can be detected in penguin chicks for up to eight weeks after hatching (Graczyk et al., 1994a; Palmer et al., 2013) and may also have protected the puffin chicks until their first mosquito season was over although it must be noted that no studies for puffins exist.

Among African penguins surviving the first infection, a decrease in the probability of mortality down to 3–4% was described (Cranfield, 2003). This phenomenon is probably based on the protective immunity in surviving individuals, that develop low-level parasitemia without showing clinical signs (Graczyk et al., 1994c, 1995). However, since infected animals are not capable of completely clearing the parasite, the bird's immune system is constantly stimulated (Grilo et al., 2016), resulting in a short and low intensity parasitemia without subsequent death when being re-infected with homologous strains of *Plasmodium* spp. (Stoskopf and Beier, 1979; Valkiūnas, 2005; Atkinson, 2008). This protective mechanism may also explain why mainly juvenile individuals died of the disease in this study. An additional factor to consider is that the maturation of the 12–15 months-old puffins' immune system might not have fully finished, increasing the risk for a lethal outcome after infection.

In contrast to case 1–3 and 5, the individuals from case 4, 6 and 7 were remarkably older at the time of death (>3 years and >12 years, respectively). High pathogenic potential based on severe burdens of exo-erythrocytic meronts in association with disease and mortality was previously reported for *P. matutinum* LINN1 (Howe et al., 2012; Dinhopf et al., 2015; Rouffaer et al., 2018; Himmel et al., 2020).

Since the effectiveness of medical treatment decreases drastically by the time the first clinical signs appear (Cranfield, 2003), it is not surprising that the puffin from 2019 (case 5) died despite being treated immediately using a combination of atovaquone and proguanil (Malarone junior®: 60 mg/kg, SID, q24h for 3 days). Due to the rapid deterioration followed by death, the animal had only received a single dose of the prescribed medication. Another factor to be considered is that although Malarone® kills tissue meronts of human *Plasmodium*, which form in hepatocytes, there is no evidence that it also destroys tissue meronts of avian *Plasmodium*, persisting in endothelial cells and macrophages. Malarone® is indeed highly effective against the erythrocytic stages of *Plasmodium* (Palinauskas et al. 2009); however, as histopathologically shown in this study, the erythrocytes are not the location, where the core of the pathogenesis is occurring. Therefore, primaquine shall be additionally considered as prevention and treatment of avian malaria since this drug is evidentially effective to kill tissue meronts of avian *Plasmodium* (Remple, 2004; Vinez et al., 2011), which may lead to relapses. Furthermore, it should be noted that posology in treatment protocols is based on empirical evidence since studies on the pharmacokinetics and pharmacodynamics of anti-malarial drugs in puffins and penguins are lacking.

Despite multiple preventive measures mentioned before, cases of fatal avian malaria have occurred at Berne Animal Park. The protective measures were implemented from the beginning of the puffin husbandry. Therefore, the effect of a single protective measure cannot be assessed. Nevertheless, it is assumed that the mortality rate would be even higher without the prophylactic measures targeting the mosquito's life cycle. Furthermore, due to the lack of a control group, the efficacy of the food additives cannot be evaluated.

For the future, metaphylactic therapy of young puffins (within the first two years of life) at Berne Animal Park using a combination of atovaquone and proguanil (Malarone junior®) and primaquine during August and September is planned.

Splenomegaly was a constant finding in all seven cases of this study, and in four individuals, hepatomegaly was observed. These lesions are consistent with gross findings in other affected puffins (Loupal and Kutzer, 1996) and penguins (Rodhain, 1939; Griner and Sheridan, 1967; Fix et al., 1988; Grim et al., 2003). In three cases (case 3, 5 and 6), a dark red discoloration of the liver was observed which can be explained by the accumulation of iron-based pigment in macrophages (Rodhain, 1939; Griner and Sheridan, 1967; Fix et al., 1988; Atkinson, 2008). The dark red-brown pigmentation of the liver originates from the accumulation of hemoglobin and is either based on the partial digestion of hemoglobin by the parasite (hemozoin as direct molecule) or the storage of iron left-overs resulting from the release of hemoglobin due to a variety of factors, including hemolysis caused by the parasite (hemosiderin as indirect molecule) during the *Plasmodium* infection. All puffins from this study showed hepatitis, four individuals additionally suffered from splenitis and two from pneumonia. Infiltration by leukocytes in various tissues is a typical finding associated with avian malaria (Rodhain, 1939; Fleischman et al., 1968; Fix et al., 1988; Silveira et al., 2013; Vanstreels et al., 2015). In this study, high burdens of tissue meronts were observed at the histopathological examination. Necrosis and inflammatory cell infiltration appear to have been caused by excessive exo-erythrocytic merogony of the parasites. High numbers of exo-erythrocytic meronts were also associated with histopathological lesions in previous studies (Dinhopl et al., 2015; Himmel et al., 2020), indicating that severe multiplication of the parasite causes deleterious effects on the host's tissues developing its high pathogenic potential.

In contrast to mammal-infecting *Plasmodium* spp. and avian-infecting *Leucocytozoon* spp., invasion of the hepatocytes is not observed in avian-infecting *Plasmodium* spp. (Valkiūnas, 2005).

The pathological changes in case 1 were moderate to severe and comparable to those described in penguins suffering from a *Plasmodium* infection. The histopathological findings in case 2 indicated a severe systemic infection with haemosporidia and the one-step multiplex PCR

revealed positive results. However, the nested PCR turned out negative. In that case, the necropsy was performed at the Animal Park, and organ samples were fixed in formalin for two days before being processed for histopathology. The discrepancy between histological findings and nested PCR result may be due to the tissue being fixed longer in formalin, causing cross-linking between protein and DNA (Chalkley and Hunter, 1975) and subsequently leading to PCR inhibition (Ben-Ezra et al., 1991) and false negative results. The combination of the positive result in the one-step-multiplex PCR and the negative result in the nested PCR may be explained by the length of both PCR products being longer in the nested reaction (377–379 vs. 524–580) and therefore more vulnerable. Since the sequence of case 2, obtained using the multiplex PCR, was absolutely identical to the sequences obtained in case 6 and 7, it is cautiously concluded that *P. matutinum* LINN1 was also the etiologic agent in case 2. In case 3, a severe infection with haemosporidia was diagnosed at the histopathological examination; however, the protozoan structures were first mistaken for *Haemoproteus* sp. A severe infection with avian haemosporidia was also detected in case 4, but it could not clearly be identified if *Plasmodium* sp. or *Haemoproteus* sp. was the etiologic agent. Case 3 and 4 highlight the difficulty to distinguish between these two genera of haemosporidia when relying only on the microscopic examination. Also, stages from other cyst-forming protozoa such as *Toxoplasma gondii* and *Sarcocystis* spp. can be mistaken for *Plasmodium*. Although a high number of haemosporidian stages were observed in several organs on histopathological examination of case 4, neither the one-step multiplex PCR for different haemosporidian species nor the nested PCR protocol for *Plasmodium* and *Haemoproteus* yielded positive results. This animal was also dissected at the Animal Park and the tissue samples were fixed in formalin for four days before being processed for histopathology what probably have led to PCR inhibition due to the same reasons that were mentioned in case 2.

Based on the histopathological findings in case 1–4, 6 and 7, the sudden death of the animals can be explained by the severe lesions caused by avian *Plasmodium*. In case 3, *Riemerella* sp. was isolated from the kidneys (data not shown), and may have contributed to weakening the bird, however the main cause of disease and cause of subsequent death was assumed to have been the *Plasmodium* infection. In case 5, an infestation by fungal structures compatible with *Aspergillus* sp. was observed beside the infection with *Plasmodium* sp. Combinations of avian malaria with other infectious diseases were already described in the past (Rodhain, 1939; Griner and Sheridan, 1967; Fleischman et al., 1968; Graczyk and Cranfield, 1996; Jencek et al., 2012; Wallace, 2014; Vanstreels et al., 2015). The mild to moderate changes associated with the *Plasmodium* infection appeared to be chronic, while the moderate to severe fungal lesions were subacute. It therefore appears that the protozoa debilitated the animal and predisposed it for fungal infection. However, it should be noted that *Aspergillus* might not elicit an initial immune response that is as strong and vigorous as the reaction initiated by *Plasmodium*; hence, the fungal infiltrate seems to take more time to exert its full impact. Moreover, based on the notoriously rapid multiplication of *P. relictum* within its host, a few days are sufficient for an exponential increase in parasite load. Consequently, it is difficult to reconstruct the infections' chronological order with absolute certainty. However, in the end the puffin's death may be explained by the inflammation due to both pathogens.

Moreover, it was shown in this study that the one-step multiplex PCR can not only be used to discriminate haemosporidia belonging to the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* in blood samples, but also in fresh tissues and in FFPE tissue sections. The sequences resulting from the one-step multiplex PCR can only be compared with sequences on GenBank, but not on the avian malaria database MalAvi what limits the exact analysis of the found haemosporidian species. Nevertheless, the one-step multiplex PCR presents an efficient method to confirm or to exclude an infection with avian haemosporidia due to the advantage that *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are analyzed in a single reaction. The nested PCR can also be used for fresh

tissue samples and DNA extracted from FFPE tissue sections, but is additionally suitable for comparison with sequences on the MalAvi database. Consequently, the nested PCR is definitely preferable for exact analytical purposes with the disadvantages of a considerably longer reaction time, a higher consumption of material and an increased risk of contamination due to the possible carry-over contamination of PCR products.

5. Conclusion

The cases of sudden death in Atlantic puffins described in this study were confirmed as avian malaria on histopathological examination, by one-step multiplex PCR and by nested PCR, and *P. relictum* lineage SGS1 and *P. matutinum* lineage LINN1 were identified as the involved etiologic agents.

This study shows that *Plasmodium* infections may also represent an important cause of death in captive puffins and should be considered as a differential diagnosis in unclear clinical or fatal cases in this threatened bird species. Further studies are warranted to improve preventive measures in captive individuals.

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Declaration of competing interest

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