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Parasitology Research
Founded as Zeitschrift für Parasitenkunde

ISSN 0932-0113
Volume 120
Number 1

Parasitol Res (2021) 120:277-288
DOI 10.1007/s00436-020-06972-1

Springer

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https://doi.org/10.1007/s00436-020-06972-1

PROTOZOOLOGY - ORIGINAL PAPER

A fatal case of a captive snowy owl (*Bubo scandiacus*) with *Haemoproteus* infection in Japan

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Received: 3 August 2020 / Accepted: 5 November 2020 / Published online: 16 November 2020

Abstract

Parasites of the genus *Haemoproteus* are vector-borne avian haemosporidia commonly found in bird species of the world. *Haemoproteus* infections are typically considered relatively benign in birds. However, some *Haemoproteus* species cause severe disease and mortality, especially for captive birds removed from their original habitat. In September 2018, a captive 15-year-old snowy owl (*Bubo scandiacus*), kept in a zoological garden of Japan, died subacutely after presenting leg dysfunction. This case showed significantly low PCV and elevated AST, ALT, CK, and LDH values. Many megalomeronts with prominent morphological characteristics of *Haemoproteus* were observed in the left leg muscles. Those megalomeronts exhibited multilocular structures and were internally filled with merozoites. A new lineage of *Haemoproteus* was detected by subsequent PCR for the cytochrome *b* (cyt*b*) gene of avian haemosporidia from DNA extracted from several organ tissues. The detected lineage was classified in the subgenus *Parahaemoproteus* and was similar to those from the wild birds inhabiting the region including the study area, suggesting that this snowy owl likely acquired its infection from wild birds. This is the first report of a fatal case of a captive bird with a locally transmitted *Haemoproteus* infection in Japan. We considered the pathogenicity of this infection in conjunction with the clinical course and hematology results. We surmise that snowy owls may be particularly susceptible to infection with *Haemoproteus* parasites, and warming northern temperatures may exacerbate the overall health of these and other high latitude birds. Further research into the prevalence of *Haemoproteus* in wild birds near zoological gardens and potential biting midge vectors is necessary for the ex situ conservation of introduced birds.

Keywords *Haemoproteus* · Snowy owl (*Bubo scandiacus*) · Pathogenicity · Japan

Introduction

Avian haemosporidia of the genus *Haemoproteus* are closely related to avian malaria parasites of the genus *Plasmodium* and are widely distributed throughout the world, including Japan. Approximately 150 species of *Haemoproteus* (Valkiūnas and Iezhova 2017) have been described, and two subgenera have been recognized according to their use of different blood-sucking dipteran insect vectors. Namely, the subgenus *Parahaemoproteus*, which includes the majority of *Haemoproteus* species, is transmitted by biting midges (Ceratopogonidae), and the subgenus of *Haemoproteus* is transmitted by hippoboscid flies (Hippoboscidae) (Valkiūnas 2005; Levin et al. 2012; Valkiūnas et al. 2013; Bukauskaite et al. 2015; Dimitrov et al. 2016). Compared with avian *Plasmodium*, *Haemoproteus* can be characterized by the absence of merogony in the host blood cells and development of meronts and megalomeronts in the endothelial cells of various

Section Editor: Leonhard Schnittger

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tissues of host birds, or myofibroblasts in certain species. The first generation of *Haemoproteus* meronts matures in the host, and the merozoites propagated there develop into the second-generation meronts known as megalomeronts. Subsequently, the merozoites produced in the matured megalomeronts invade the erythrocytes and develop into gametocytes (Atkinson et al. 1986). These meronts and megalomeronts have been observed in various tissues, mostly lung, liver, spleen, kidneys, heart, and skeletal musculature, and in brain, tongue, proventriculus, ventriculus, and bone marrow (Earle et al. 1993; Cardona et al. 2002; Fukui et al. 2002; Valkiūnas 2005; Donovan et al. 2008; Valkiūnas and Iezhova 2017; Groff et al. 2019; Metwally et al. 2019; Ortiz-Catedral et al. 2019). This exo-erythrocytic stage in the life cycle of *Haemoproteus* may cause tissue degeneration in the bird's organs (Valkiūnas and Iezhova 2017), occasionally leading to severe organ dysfunction or necrosis. Those infected individual birds with meronts and megalomeronts in the tissues also can have hepatomegaly, hepatitis, hepatic hemorrhage, and hepatic necrosis (Evans and Otter 1998; Donovan et al. 2008; Tostes et al. 2015; Lee et al. 2018; Groff et al. 2019), associated with the parasite development, as well as degeneration such as hemosiderin deposition in the spleen (Cardona et al. 2002; Fukui et al. 2002). In addition, tissue hemorrhage and myositis in skeletal muscles, myocardium, or ventriculus have been reported (Earle et al. 1993; Evans and Otter 1998; Cardona et al. 2002; Donovan et al. 2008; Ortiz-Catedral et al. 2019).

Haemoproteus infections have typically been considered relatively benign in infected birds. Many species of avian haemosporidian have been found to remain as chronic infections until the end of the host's lifespan (Valkiūnas 2005) and this has been also demonstrated in *Haemoproteus*-infected birds in controlled laboratory conditions (Valkiūnas and Iezhova 2017). However, severe and/or fatal pathogenicity in captive *Haemoproteus*-infected birds has been reported in various bird species (Cardona et al. 2002; Tarello 2005; Donovan et al. 2008; Kakogawa et al. 2019). *Haemoproteus* outbreaks have occurred in aviaries in European countries, resulting in the death of captive 20 parrots of 12 species native to Australasia and a monk parakeet (*Myiopsitta monachus*) native to South America (Ortiz-Catedral et al. 2019). Meanwhile, as in a recent study of a white-headed woodpecker (*Dryobates albolarvatus*) suspected of dying from *Haemoproteus* infection (Groff et al. 2019), mortality related to this infection has been reported in wild birds. *Haemoproteus* has been also detected in raptors (Apanius and Kirkpatrick 1988; Pérez-Rodríguez et al. 2013; Tostes et al. 2015) including the order Strigiformes (Bishop and Bennett 1989; Hisada et al. 2004; Karadjian et al. 2013, 2014; Pornpanom et al. 2019) with severe clinical signs in some cases (Fukui et al. 2002; Tarello 2007; Niedringhaus et al. 2018). Those affected individuals often exhibit clinical signs such as anorexia, lethargy, ataxia, vomiting, or pallor of skin and mucous membranes, i.e., a clinical sign of anemia (Cardona et al. 2002; Tarello 2005, 2007; Tostes et al. 2015; Lee et al. 2018). However, sudden death with no clinical signs can occur (Earle et al. 1993; Donovan et al. 2008; Niedringhaus et al. 2018). Several studies of Strigiformes birds suggested that snowy owls (*Bubo scandiacus*) are highly susceptible to *Haemoproteus* infection. Most *Haemoproteus*-infected snowy owls presented anorexia, depression, or an anemic condition, and sudden death or acute fatal cases have been reported (Evans and Otter 1998; Mutlow and Forbes 2000; Baker et al. 2018; Lee et al. 2018). For example, five young captive snowy owls in Cumbria, UK, showed clinical signs including pale mucus membranes and severe regenerative anemia, and all but one died. The lowest PCV (packed cell volume) value was 5% among them (Mutlow and Forbes 2000).

The wild snowy owl has a circumpolar distribution and breeds mainly in the Arctic tundra (Marthinsen et al. 2009). In the nonbreeding season (winter), many snowy owls migrate further south into the USA, northern Europe, and north Asia (Holt et al. 2016; BirdLife International 2017) including northern Hokkaido, Japan (Kosugi et al. 2013). Snowy owls are listed as a vulnerable species because they are rapidly declining in North America, and possibly Northern Europe and Russia (BirdLife International 2017). Global climate change may have a significant impact upon the start of spring and snowmelt in the breeding areas and affect the population cycles of rodents, which are the main prey of snowy owls. This may be a cause of the rapid decline of the wild snowy owl population (BirdLife International 2017; Westrip 2017). As an additional note, no direct human factors have been reported to have a significant impact on the abundance of snowy owls (BirdLife International 2017), but recently, the influence of the movies has increased their popularity as pets, and the accompanying illegal trade threatens wild snowy owls (Siriwat et al. 2020). Meanwhile, it is a popular species in exhibitions and frequently kept in zoological institutions (Wills et al. 2016). According to the Japanese Association of Zoos and Aquariums (https://www.jaza.jp), about 43% of the member zoos exhibit snowy owls. The observed lethal case in this study with a *Haemoproteus* infection raised concerns regarding the protection and management of captive birds, particularly rare species in ex situ situations like zoos and aviaries.

A captive snowy owl was found to be dead subacutely after presenting clinical signs such as an apparent left leg dysfunction in a zoological garden of Japan in 2018. Subsequent histopathological and molecular biological examinations revealed that this individual was infected with *Haemoproteus* parasites. Here, we report on the pathogenicity of this *Haemoproteus*-infected case from various perspectives, along with the clinical course and hematology results.

Materials and methods

Case history

The snowy owl in this case was a 15-year-old male, born, and raised in a zoological garden, Tobu Zoo in Saitama Prefecture, Japan (36°01′12.3″N 139°43′11.7″E). The first clinical manifestation of anorexia was observed in September 2018. The body weight at this time was 1256 g. On the fifth day after the onset of anorexia, the bird presented left leg lameness, standing on the right leg instead. In addition, the bird was often observed resting on the sternum, or standing on the metatarsus rather than the foot. Radiographic examination was performed on the same day, but no abnormality was found. On the eighth day, this owl did not maintain its normal correct posture, but had the left leg extended. No abnormalities were found in direct and flotation examination of feces. Twenty milliliters of subcutaneous fluid was administered. On the ninth day, blood samples were collected for hematological examinations. The owl was treated orally with 12.5 mg/kg enrofloxacin, 10.5 mg/kg carprofen, and 83.5 mg/kg glutathione. The body weight was 1180 g. On the tenth day, vomiting occurred, so that 10 mg/kg enrofloxacin, 5 mg/kg carprofen, 167 mg/kg glutathione, and 0.5 mg/kg metoclopramide were given intramuscularly. This snowy owl was found dead on the morning of the eleventh day. At necropsy, mild hepatomegaly was observed.

Hematological examinations

Two days before the death of the snowy owl, a blood sample was collected from the basilic vein under manual restraint without administration of sedatives or anesthetics. The packed cell volume (PCV) was measured in a hematocrit tube after centrifugation. Plasma biochemistry test was performed for total protein, albumin, globulin, glucose and uric acid, the enzymatic activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and creatine kinase (CK), by a clinical chemistry analyzer (DRI-CHEM NX500V, FUJIFILM, Japan). Note that no blood smears were prepared, and thus, we were unable to use microscopy to study the blood stages of the parasite.

Histopathological examinations

The brain, heart, lungs, liver, spleen, kidneys, testicles, proventriculus, ventriculus, a part of intestinal tract, uropygial gland, and skeletal muscles of left leg were collected and were fixed in 10% neutral-buffered formalin. Representative trimmed tissues were routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (HE). HE slides were reviewed by Dr. Hirotaka Kondo, veterinary pathologist certified by American College of Veterinary Pathologists.

Molecular screening and phylogenetic analyses

Total DNA was extracted from the blood collected 2 days before the death and the tissues of liver, spleen, and heart, also from a blood clot collected at necropsy, using a conventional phenol/chloroform/isoamyl alcohol (PCI) extraction method. The extracted DNA was screened by nested polymerase chain reaction (PCR) for avian haemosporidia DNA of the partial mitochondrial cytochrome *b* (cyt*b*) gene with the first PCR primer set of HaemNFI-HaemNR3 (Hellgren et al. 2004), then the second primer sets of HaemNF-HaemNR2 (Waldenström et al. 2004) for *Plasmodium* and *Haemoproteus* and HaemFL-HaemR2L (Hellgren et al. 2004) for *Leucocytozoon*, respectively. All PCR conditions were followed as described previously (Hellgren et al. 2004). The PCR products were resolved on 1.5% agarose gels stained with ethidium bromide and visualized under ultraviolet light. The amplified products were purified using the Monarch DNA Gel Extraction Kit (New England Biolabs Japan) and sequenced at GENEWIZ Japan (Kawaguchi, Saitama, Japan) using the second PCR primer set. Obtained sequences were trimmed to match the 479-bp standard cyt*b* region using ApE (A plasmid Editor) software version 2.0.48 by M. Wayne Davis (https://jorgensen.biology.utah.edu/wayned/ape/), then were compared with sequences in the GenBank database using Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/) and the MalAvi (Bensch et al. 2009) database. Identified sequences were aligned with Clustal W program, and phylogenetic analyses of the 458-bp cyt*b* gene sequences were performed using the maximum likelihood (ML) method based on the general time reversible model, including the gamma distribution with invariant sites using the program of MEGA X software (Kumar et al. 2018). A total of 1000 cycles of bootstrap resampling were performed to assess tree topology.

Results

Hematological examinations

Obtained hematological values are shown in Table 1. PCV of this case was significantly lower and plasma AST, LDH, and CK enzyme activity were higher than the blood test reference value of snowy owls (n = 11) (Ammersbach et al. 2015a, b). ALT enzyme activity was also higher than the reference value of great horned owls (*Bubo virginianus*) (n = 10) (Samour 2006), but no reference values were found for snowy owls. Other parameters mostly remained comparable with those reference value ranges.

Table 1 Results of hematological examinations of the snowy owl (*Bubo scandiacus*). PCV showed significantly lower values, and AST, ALT, LDH, and CK were higher than the reference values

Analytes (units)	Values	Reference values of snowy owls (n = 11)[b]	
		Median	Range
PCV (%)	17	50	37–60
Total protein (g/L)	39	38	29–48
Albumin (g/L)	14	34	20–39
Globulin (g/L)	25	3	0–24
AG ratio	0.56	0.8	0.4–1.3
Glucose (mmol/L)	16.8	18.3	12.0–25.6
AST (U/L)	> 1000[a]	262	186–310
ALT (U/L)	843	39[c]	-
LDH (U/L)	> 900[a]	209	132–861
Uric acid (μmol/L)	589	677	306–1438
CK (U/L)	> 2000[a]	213	20–3246

PCV packed cell volume, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *CK* creatine kinase

[a] Value above measurable upper limit

[b] Reference from Ammersbach et al. 2015a, 2015b

[c] Reference value of great horned owls (n = 10) (Samour 2006)

Histopathological views

Numerous cystic protozoan structures of megalomeronts full of merozoites were observed in the skeletal muscle fibers of the left leg (Fig. 1). The megalomeront had a thick eosinophilic outer wall separated with internal septae. There was moderate infiltration of eosinophils and/or heterophils and histiocytes in the interstitium. The lesions were histopathologically diagnosed as eosinophilic and/or heterophilic and histiocytic myositis with intramuscular protozoan megalomeronts. The nuclei of host cells are not enlarged either by meronts or megalomeronts of *Haemoproteus* species, and thus, the infection is clearly different from *Leucocytozoon* species (Valkiūnas and Iezhova 2017). The megalomeronts in the present study did not enlarge the nuclei of host cells. Accordingly, the observed feature indicated that these were megalomeronts of *Haemoproteus* species.

In the liver, the portal areas were multifocally expanded by moderate numbers of lymphocytes and plasma cells. The sinusoids were dilated and congested. Other representative lesions included mild hemosiderosis in the spleen, mild lymphoplasmacytic enteritis, and testicular degeneration. No significantly unusual lesions were observed in the brain, heart, lungs, kidneys, proventriculus, ventriculus, nor uropygial gland. In addition, we found no parasitized erythrocytes in the observed blood vessels on the HE slides.

The structures of observed megalomeronts

The megalomeronts observed in the skeletal muscle tissue of the leg in this study are covered with a thick capsular-like wall and mostly show multilocular structures with septae (Fig. 1). However, some are not divided into compartments, which may be due to differences in the growth stages of megalomeronts, or may be dependent on tissue section sites. Similar features have been observed in megalomeronts in the skeletal and cardiac muscles of a bleeding heart dove (*Gallicolumba luzonica*) infected with what might be *H. columbae* (Earle et al. 1993) and in the cardiac muscle of a red-crowned parakeet (*Cyanoramphus novaezelandiae*) (Himmel et al. 2019) infected with *H. minutus*. In particular, the structures of *Haemoproteus* megalomeronts in the ventriculus of a budgerigar (*Melopsittacus undulatus*) (Harris and Gardiner 2017) are very similar to those of this study. The multilocular structure of the megalomeront was once thought to be also found in *Leucocytozoon* species, but currently seems to be recognized as a structure particular to *Haemoproteus* species (Paperna and Gill 2003; Himmel et al. 2019). In addition, megalomeronts of *Haemoproteus* are often adjacent to each other and form large groups (Valkiūnas and Iezhova 2017), but in the present study, they are seen individually and no groups have been observed anywhere on the histopathological slides.

A large number of merozoites were observed in each compartment of the detected megalomeronts, and vacuole areas are also seen there. No well-defined or thin-membrane-covered cytomeres were visible, as seen in megalomeronts in the liver of a magnificent bird of paradise (*Diphyllodes magnificus hunsteini*) infected with *H.* sp. (Donovan et al. 2008) or in the liver of a house sparrow (*Passer domesticus*) infected with *H. passeris* (Valkiūnas and Iezhova 2017). The expression pattern of these merozoites appears to be chromatin aggregates found in mature megalomeronts (Duc et al. 2020), but some resemble the irregular forms of cytomeres found in megalomeronts in lungs of the European robin (*Erithacus rubecula*) infected with *H. attenuatus* (Valkiūnas and Iezhova 2017). Merozoite nuclei that exist along the inside of the outer wall or septae also can be seen.

Molecular detection and phylogenetic analysis for avian haemosporidia

The nested PCR using the primer set of HaemNF-HaemNR2 followed by sequencing detected the identical nucleotide sequence in all the examined DNA samples of blood (ante and post mortem), tissues of liver, spleen, and heart. No double peaks were found in the sequencing chromatograms from any of the samples. In addition, no amplification was observed for *Leucocytozoon*. The obtained sequence was not identical to any known lineages deposited in the databases. It was assigned the

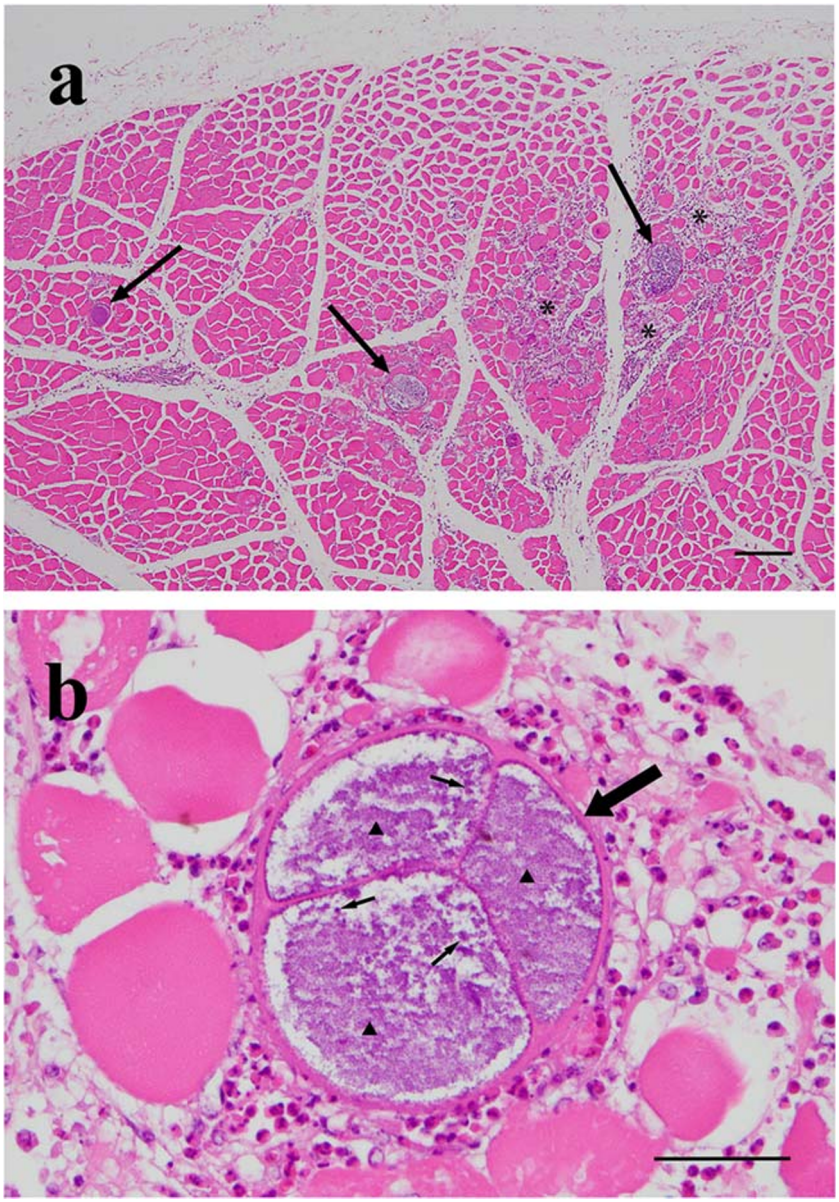

Fig. 1 Megalomeronts of *Haemoproteus* sp. observed in the skeletal muscle tissue of the left leg of the snowy owl. Megalomeronts (arrows) developed in the skeletal muscle fibers. Some parts of skeletal muscle fiber are degenerate and necrotic, and the infiltration of inflammatory cells surrounding the fibers is seen (areas of asterisk) (**a**). The megalomeront has a roundish and multilocular structure with a diameter of 100–150 μm and is covered with a thick capsular-like wall (large arrow). A myriad of basophilic-stained merozoites (areas of triangle mark) and irregular shaped cytomere-like specks (small arrows) are observed in each compartment separated by septae (**b**). Hematoxylin-eosin stain. *Scale bars*; **a** 200 μm, **b** 50 μm

lineage name for the MalAvi database BUBSCA02, with the GenBank accession number LC535804.

Phylogenetic analysis showed that this new lineage was classified as genus *Haemoproteus* and it belonged to the clade of subgenus *Parahaemoproteus*. *H. noctuae* CIRCUM01 and three lineages of *H. syrnii* are grouped in the same clade as the present lineage, and the most closely related lineage was *H. noctuae* CIRCUM01 with nucleotide similarity of 98.75% (473/479bp) (Fig. 2). *H. noctuae* and *H. syrnii* are two known widespread *Haemoproteus* species infecting birds of the order Strigiformes (Bishop and Bennett 1989; Valkiūnas 2005; Bukauskaité et al. 2015). In addition, it was classified into the same clade as several *Haemoproteus* lineages detected from wild birds inhabiting the southern Kanto region of Japan (Tokyo, Saitama, Kanagawa, and Chiba Prefectures) in recent years (Table 2). New data have been added to the data from a previous study (Inumaru et al. 2017) in Table 2. These new data were obtained from blood samples of rescued wild birds in the facilities located in Chiba Prefecture and Kanagawa Prefecture. Table 2 also includes a blood sample of a red-flanked bluetail (*Tarsiger cyanurus*) collected using mist nets for epidemiologic investigation of blood parasites in the Nihon University experimental forest (Kanagawa Prefecture). These samples were analyzed using the same methods as in this study, or with the second PCR primer set, HaemF-HaemR2 (Bensch et al. 2000).

Discussion

In the fatal case of this captive snowy owl, we detected a new molecular lineage of *Haemoproteus* sp. from the

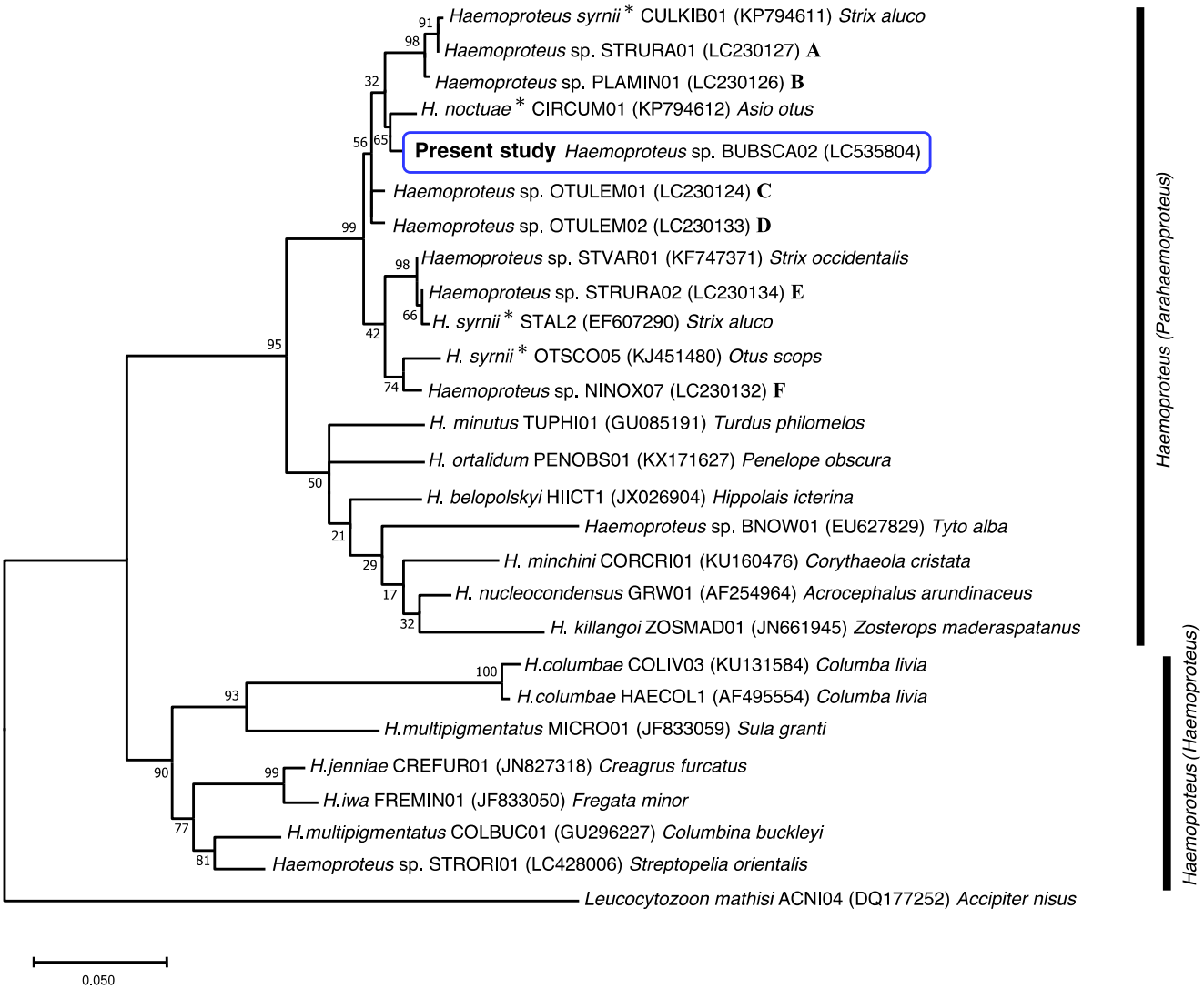

Fig. 2 Maximum-likelihood phylogenetic analysis of the detected *Haemoproteus* lineage from a snowy owl based on a portion of the mitochondrial cytochrome *b* gene (458 bp) compared with other *Haemoproteus* spp. *Leucocytozoon* sp. is used as the outgroup. GenBank accession numbers and host bird species are given after parasite lineage names from MalAvi database. The asterisks indicate the *H. noctuae* or *H. syrnii* lineages of which natural hosts are birds of the order Strigiformes. The present lineage is classified in the same clade as the lineages detected from wild birds in the southern Kanto region of Japan, detailed in A-F in Table 2

blood and the tissues and found many megalomeronts that had developed in the skeletal muscle fibers of the left leg. Some cases of *Haemoproteus* infection in similar introduced and captive birds have been reported in Japan (Kakogawa et al. 2019; Inumaru et al. 2020). In addition, several *Haemoproteus* spp. have also been detected in wild birds of Japan (Murata 2002; Imura et al. 2012; Yoshimura et al. 2014; Inumaru et al. 2017), but no pathogenicity has been reported in these native birds. As discussed below, this study considers the correlation between clinical signs, histopathological and hematological results, and pathogenicity of *Haemoproteus* infection. We also infer the probable transmission and origin of this infection.

Inferred origin of infection of the detected *Haemoproteus*

The detected *Haemoproteus* sp. was grouped into the same clade as those identified from wild birds inhabiting the region including this zoological garden (Fig. 2). *H. noctuae* CIRCUM01 from the long-eared owl (*Asio otus*) in Russia is found to be a closely related lineage, but has not been detected from birds in Japan. However, two lineages of *Haemoproteus* sp. OTULEM01 with nucleotide similarity of 98.75% (473/479 bp) and OTULEM02 with nucleotide similarity of 98.47% (451/458 bp) infecting owls in Japan are also phylogenetically close to the detected lineage. Moreover, the present and other lineages from Japanese owls in the southern Kanto region were classified into the subgenus

Table 2 Details of *Haemoproteus* lineages detected from wild birds in the southern Kanto region of Japan from 2014 to 2018 shown in Fig. 2 (A–F). New data are indicated with an asterisk (*) which have been added since a previous study (Inumaru et al. 2017)

Group (lineage)	Species of avian host	Detection year	Location
A (STRURA01)	Ural owl (*Strix uralensis*)	2014	Chiba
B (PLAMIN01)	Bull-headed shrike (*Lanius bucephalus*)	2014	Chiba
	Eurasian tree sparrow (*Passer montanus*)	2014	Chiba
	Black-faced spoonbill (*Platalea minor*)	2015	Chiba
	Ural owl (*Strix uralensis*)	2015	Chiba
	White-cheeked starling (*Sturnus cineraceus*) *	2016	Chiba
	Ural owl (*Strix uralensis*) *	2017	Chiba
C (OTULEM01)	Eurasian woodcock (*Scolopax rusticola*)	2014	Tokyo
	Grey heron (*Ardea cinerea*)	2014	Tokyo
	Sunda scops owl (*Otus lempiji*) *	2014	Kanagawa
	Sunda scops owl (*Otus lempiji*)	2015	Chiba
	Meadow bunting (*Emberiza cioides*) *	2017	Chiba
D (OTULEM02)	Sunda scops owl (*Otus lempiji*)	2016	Kanagawa
E (STRURA02)	Barn swallow (*Hirundo rustica*)	2014	Chiba
	Red-flanked bluetail (*Tarsiger cyanurus*) *	2015	Kanagawa
	Ural owl (*Strix uralensis*)	2015	Chiba
	Ural owl (*Strix uralensis*) *	2018	Chiba
	Ural owl (*Strix uralensis*) *	2018	Kanagawa
F (NINOX07)	Brown hawk-owl (*Ninox scutulata*)	2014	Tokyo

Parahaemoproteus, suggesting that they are transmitted by biting midges (Bukauskaité et al. 2015). Biting midges are widely distributed in Japan including at the study area of the Saitama Prefecture (Wada 1999). This individual was bred at this zoological garden until its death, without any relocations throughout its life, suggesting that there was no chance to be exposed to the vectors outside the zoo area. Thus, this snowy owl was likely to have been infected with *Haemoproteus* from the native birds in the vicinity of this zoological garden by biting midges.

Clinical features and pathological effects by infection

This infected snowy owl showed several clinical signs like anorexia, vomiting, and a striking dysfunction of the left leg. Many megalomeronts specific to *Haemoproteus* developmental stages in the host tissues were observed in the left leg skeletal muscles, and the lesions were diagnosed as myositis with intramuscular megalomeronts. The high muscle-specific enzyme CK value in the blood also indicates that the skeletal muscle was significantly damaged. Consequent radiographic examination, visual inspection, and palpation revealed no obvious abnormalities, suggesting that fractures, traumas, and severe sprains of the left leg could be excluded. Our findings suggested that the leg dysfunction was due to myositis caused by the occurrence of megalomeronts. Similar to this study, domestic turkeys experimentally infected with *Haemoproteus mansoni* had megalomeronts with myositis in the legs, and most turkeys in the high-dose group became lame in one or both legs (Atkinson et al. 1988). Therefore, *Haemoproteus* infection should be considered as one of the causes of musculoskeletal abnormalities such as lameness that are not diagnosed by radiography or other examinations. The megalomeront associated myositis in the leg skeletal muscle was also found in a *Haemoproteus*-infected bobwhite quail (*Colinus virginianus*) (Cardona et al. 2002). Most of the *Haemoproteus*-infected snowy owls showed anorexia as in this case, with lethargy and depression (Evans and Otter 1998; Mutlow and Forbes 2000; Lee et al. 2018). Another infected snowy owl died of a sudden cardiopulmonary arrest without clinical signs (Baker et al. 2018).

Hematological features

The remarkably lower PCV (17%) of this case indicates that this snowy owl was severely anemic when compared with normal bird PCV ranges (35~55%) (Campbell 1994). Generally, infected erythrocytes are rapidly cleared from the blood circulation by the reticuloendothelial system of the spleen, liver, bone marrow, and some other organs, and thus in the presence of a large number of infected erythrocytes (= during high parasitemia), hematopoiesis cannot compensate for the losses of erythrocytes, resulting in acute anemia (Valkiūnas 2005). However, not following this pattern, in an infection experiment with *Haemoproteus mansoni*, the infected turkeys did not develop anemia despite high (over 50%) parasitemia (Atkinson et al. 1988). In the present study, infected blood cells were not identified on the HE-stained pathological slides. However, this study does not provide information on the blood stages of the *Haemoproteus* sp. and parasitemia

that would be obtained from blood smears. Therefore, it is not possible to consider whether the severe anemia of the snowy owl is directly associated with the *Haemoproteus* infection. As with the case of the snowy owls in Cumbria, UK, mentioned above, anemia has certainly developed in other *Haemoproteus*-infected birds (Evans and Otter 1998; Tostes et al. 2015; Lee et al. 2018). The development of anemia during *Haemoproteus* infection is less pronounced than *Plasmodium* or *Leucocytozoon*, but it may increase in severity due to other factors from the host's side (Valkiūnas 2005). Other causes of anemia to be considered, besides *Haemoproteus* infection, should include stresses, illnesses, other pathogen infections of host, or their duplication. In other words, although expected to be related, it is difficult to conclude that the anemia in this case was directly due to *Haemoproteus* infection.

AST and ALT in birds are considered nonspecific because they are found in various organs, but AST is considered to be a highly sensitive indicator of hepatocellular disease (Harr 2002), and ALT activity increases with damage to hepatocytes (Vashist et al. 2011). LDH is found particularly in skeletal muscles, heart, liver, kidneys, bones, and erythrocytes, and elevated activity of this enzyme is observed due to liver and muscle damage or hemolysis (Hochleithner 1994). Thus, these blood enzyme activities are considered to reflect damage to various organs and tissues. CK can be found not only in skeletal muscle as previously mentioned, but also in all muscular structures such as cardiac muscle and muscles of the gastrointestinal tract (Harr 2006). The high values of AST, ALT, LDH, and CK detected here reveal that the damage of skeletal muscles and organs including the liver, spleen, and intestinal tract are consistent with the histopathological results. Also, all or some of these enzymatic activities have been found to be high in *Haemoproteus*-infected birds (Evans and Otter 1998; Borji et al. 2011; Tostes et al. 2015; Metwally et al. 2019). It is thought that the mode of infection of *Haemoproteus* that invades multiple organs, muscle tissues, and erythrocytes at the same time results in a simultaneous increase in the enzymatic activities of AST, ALT, LDH, and CK. Anorexia, lethargy, ataxia, or vomiting, which are often found in *Haemoproteus*-infected birds as described above, can also be common in sick birds. So, these clinical signs cannot be differential diagnostic factors for *Haemoproteus* infection. The standard for confirming the infection of *Haemoproteus* in a living bird is to detect infected erythrocytes on a blood smear, but particularly fatal cases undergo a rapid course and are usually diagnosed by necropsy, histopathological examination, or subsequent PCR (Himmel et al. 2019), and therefore, it may not be possible to prepare a complete blood smear after the bird has died, as in this study. Additionally, infections do not always result in infected erythrocytes that would be visible on blood smears, because *Haemoproteus* does not reach the blood stages in the case of incomplete development in non-adapted hosts (Valkiūnas et al. 2017; Valkiūnas and Iezhova 2017). Such low PCV and high AST, ALT, LDH, and CK levels revealed by hematological examination might be used as parameters to suspect *Haemoproteus* infection as one of the causes of illness and would suggest the need for diagnostic tests such as blood smears or PCR. However, there seems to be no specific preference for the organs and tissues for the development of the megalomeronts with some exceptions (Valkiūnas and Iezhova 2017). As different *Haemoproteus* species might infect different organs, it is necessary to consider that the results of blood biochemistry are expected to vary accordingly.

Impact of *Haemoproteus* infections on the conservation of snowy owls

Up to the present, no natural infection cases of *Haemoproteus* in wild snowy owls have been reported. They inhabit the Arctic regions in North America and the Palearctic, which may lack suitable vectors, or the environmental temperature may be too low for the completion of the *Haemoproteus* life cycle in vectors (Mutlow and Forbes 2000; Ramey et al. 2014). However, it has been reported that warming and changes in vegetation in the Arctic caused by climate change bring new pathogens to the wildlife inhabiting the area (Altizer et al. 2013). Also, areas of high avian haemosporidian prevalence might expand because insect vectors distributions expand with warmer temperatures, and blood parasites previously not adapted to environmental conditions could infect northern birds (Loiseau et al. 2012; Van Hemert et al. 2014; Sehgal 2015; Caminade et al. 2019).

On the other hand, in captive snowy owls, including individuals rescued and kept for a while, many severe cases associated with *Haemoproteus* infection have been reported (Evans and Otter 1998; Mutlow and Forbes 2000; Baker et al. 2018; Lee et al. 2018). This owl species can be considered as a potentially naïve host to vector-borne parasite infections. *Haemoproteus* parasites are less host specific than previously thought: some lineages have been found to readily switch or share hosts (Waldenström et al. 2002; Križanauskienė et al. 2006; Donovan et al. 2008; Inumaru et al. 2020). So, non-native birds might be much more susceptible to pathogens not endemic to their original environment (Mutlow and Forbes 2000; Donovan et al. 2008; Ortiz-Catedral et al. 2019).

The lineage identified here was closely related to *H. noctuae* as mentioned above. This *Haemoproteus* species was first described by Celli and Sanfelice in a little owl (*Athene noctua*) in Italy, 1891 (Bishop and Bennett 1989). Since then, it has been confirmed in all zoogeographical regions, except the Antarctic, but with no records north of the Arctic circle. More than 30 species of Strigiformes belonging to families Strigidae and Tytonidae are natural hosts of

H. noctuae (Valkiūnas 2005). Nevertheless, a captive snowy owl infected with *H. noctuae* showed severe pathogenicity with a simultaneous *Leucocytozoon* infection (Evans and Otter 1998). Therefore, the detected lineage might be also highly pathogenic to snowy owls.

In addition to the above case of superinfection, fatal cases of captive snowy owl suspected of having reduced immunity due to co-infection of *Plasmodium* or *Leucocytozoon* and facilitating the establishment of West Nile virus (WNV) infection have been reported (Harasym 2008). In another case, a snowy owl infected with *Haemoproteus*, *Leucocytozoon*, and *Plasmodium* died after moving to a new environment. It was concluded that the cause of death was a complication of WNV infection discovered after death and hemoparasitic infections (Baker et al. 2018). These cases suggest that the pathogenicity of avian hemoparasite infections is facilitated by superinfection and stress on the host, or may trigger the development of other diseases. In other words, the onset of *Haemoproteus* infection can depend on the degree of immune activity of the host birds (Gonzalez et al. 1999; Navarro et al. 2003). It may also be related to the many serious consequences of infected juveniles or youth with immature immune systems (Evans and Otter 1998; Mutlow and Forbes 2000; Tarello 2005; Baker et al. 2018; Niedringhaus et al. 2018; Groff et al. 2019). Thus, it is quite possible that the death of the snowy owl in this study was also influenced by other factors besides the *Haemoproteus* infection. Many parameters of host status such as age, physical condition, and living environment can be important factors in considering the infection risks of avian haemosporidia for captive birds particularly non-native species.

Conclusion

The observed pathogenic phenotypes such as anemia, leg dysfunction, and the results of hematological and histopathological examinations were correlated and consistent with the observed *Haemoproteus* infection. Phylogenetic analysis of the detected *Haemoproteus* lineage suggested that this owl was likely infected from wild birds in the vicinity of this study site. This study reported the first fatal case of a captive snowy owl born in a zoological garden of Japan with a locally transmitted *Haemoproteus* infection. This bird species is distributed in Arctic regions where suitable vector species may be absent or the temperature too low for *Haemoproteus* species to complete their development. Here, we note that introduced birds naïve to domestic pathogens might be particularly susceptible to infections. In order to prevent the spread of *Haemoproteus* infections, further studies on the prevalence of parasites in captive birds and native wild birds, and also controlling of biting midge populations, would be essential for effective ex situ conservation.

Acknowledgments We would like to thank the staff of Tobu Zoo for helping the sample collection.

Authors' contributions The study concept and design were performed by YS and MY. KO collected all samples. MY conducted molecular analysis. HK carried out histopathological diagnosis. HS and YE helped conducting histopathological diagnosis and molecular experiments. YS and RS supervised the project. MY, HK, YS, and RS wrote the manuscript. All authors read and approved the final manuscript.

Funding This study was partially supported by the Grant-in-Aid for Scientific Research (KAKENHI nos. 26450484) from the Japan Society for the Promotion of Science (JSPS).

Compliance with ethical standards

All procedures for collecting samples from bird were performed in accordance with the ethical standards of the Act on Welfare and Management of Animals 1973, Japan.

Conflict of interest The authors declare that they have no conflict of interest.

Research involving animals All procedures for collecting samples from this bird were performed in accordance with the ethical standards of the Act on Welfare and Management of Animals 1973, Japan.

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