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The Difficulties of Ex Situ Conservation: A Nationwide Investigation of Avian Haemosporidia Among Captive Penguins in Japan

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Abstract: Avian malaria has been a continuous problem in both wild and captive populations of penguins throughout the world. In Japan, where there are over 3000 captive penguins, avian malaria (by *Plasmodium* spp.) and haemoproteosis (by *Haemoproteus* spp.) have been sporadically detected throughout the country. However, no comprehensive studies have been carried out, and the national status of infection has been unknown until now. In this study, the prevalence and lineage composition of haemosporidian parasites was investigated in captive penguins throughout Japan for the first time. A total of 1203 penguins from 55 facilities were sampled from January 2010 to December 2019. Parasites were detected by nested PCR and microscopy of blood and tissue samples. The total prevalence was 7.48% for *Plasmodium* and 1.75% for *Haemoproteus*, of which some are suggested to have been acquired during the study period. The odds of infection were higher in individuals kept outdoors compared to indoors, re-confirming that exposure to vectors is one of the major factors. Additionally, the odds of death were higher in infected individuals, although differences between parasite lineages were also observed. This study provides an overview of avian malaria in penguins of Japan in the hope of guiding future studies and conservation actions in captivity.

Keywords: avian malaria; *Haemoproteus*; haemosporidia; Japan; national survey; penguin; *Plasmodium*; prevalence

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1. Introduction

Avian malaria is a vector-borne disease caused by *Plasmodium* parasites. Along with closely related haemosporidian parasites of the genus *Haemoproteus* and *Leucocytozoon*, these parasites are distributed throughout the world [1]. These avian haemosporidian parasites can cause varying levels of pathogenicity in host birds depending on the parasite species and host species. Infection can sometimes be extremely pathogenic for some avian hosts, especially if the host species have historically had little to no exposure to these pathogens [1–3]. In Hawaii, numerous endemic honeycreepers went extinct due to the

introduction of avian malaria and avian poxvirus, which are both pathogens that had not been native in Hawaiian islands historically [2,4,5]. Similarly, cases of death due to avian haemosporidia have been reported from many captive birds outside of their natural distribution, including cranes [6], parrots [7,8], passerine birds [9], owls [10,11], and penguins [12,13].

Many species of penguins are currently endangered due to human-caused threats such as pollution, climate change, habitat loss, and infectious diseases [14]. To conserve these endangered birds, populations in captivity have been established throughout the world. While species of the Antarctic or sub-Antarctic region are generally kept indoors, temperate species are often kept in outdoor enclosures where vector insects such as mosquitoes and biting midges can freely access the birds [15]. These penguins are therefore constantly exposed to vectors that can transmit avian haemosporidia from wild birds of surrounding areas [1,16–18]. As most penguin species naturally inhabit areas free of avian haemosporidia or areas with low prevalence, they are extremely sensitive to these pathogens. Consequently, many fatal cases due to avian haemosporidia, particularly avian malaria, have been reported [12,16,19–21]. Avian haemosporidia are now considered one of the most significant pathogens in the conservation of penguins [12,22–24].

In Japan, there are over 3000 penguins in zoos and aquariums across the nation [25,26]. The first report of avian haemosporidia in captive penguins of Japan was from Magellanic penguins (*Spheniscus magellanicus*) of Mie Prefecture in 1987 [27]. Since then, avian malaria (*Plasmodium* spp.) and haemoproteosis (*Haemoproteus* spp.) have been sporadically detected throughout Japan [19,20,28,29]. Furthermore, avian malaria has been detected from *Culex pipiens* group mosquitoes caught at zoological facilities, including individuals that had fed on penguins and wild birds [17,29,30]. These reports strongly suggest that avian haemosporidia are actively transmitted at such facilities in Japan between captive penguins and wild birds that inhabit the surrounding areas. However, the prevalence and lineage composition, which are important for considering conservation actions, of avian haemosporidia in penguins across Japan were unknown. The prevalence and lineage composition of haemosporidian parasites in captive penguins throughout the country were investigated in this study. The environment of the enclosure and vital status were incorporated in the analysis to reveal possible risk factors for ex situ populations of endangered penguins.

2. Materials and Methods

2.1. Sample Collection

Whole blood from captive penguins was sampled between January 2010 and December 2019 at 55 facilities of 29 prefectures across Japan. Note that individuals from two previously reported studies are included as part of this study [28,29]. The environment in which these penguins were kept differed by species and facility. Each individual was classified into two environmental groups: outdoor enclosure (outdoor group) and indoor enclosure (indoor group). The indoor group included individuals that were maintained indoors but allowed outdoors during the winter months (November to April). All deceased individuals belonged to the outdoor group. Two Humboldt penguins (*Spheniscus humboldti*) passed away during the study period and were included in the deceased group. Although information including relocation history between facilities, prophylactic medication history, symptoms and treatments during sampling were provided by some facilities, not all individuals were provided with this information.

In live penguins, blood was obtained from either the brachial or metatarsal vein. Some individuals were sampled multiple times mainly for routine medical check-ups, although the frequency and span of sampling differed between individuals and facilities. In penguins that died, a small portion of organ tissue such as liver, spleen and lung was

obtained. All samples were kept in microtubes with 70% ethanol. Blood smears were prepared when possible.

All samples and blood smears were then sent to the Laboratory of Biomedical Science of Nihon University College of Bioresource Sciences. The blood and tissue samples were kept at −20 °C until DNA isolation. Blood smears were fixed with 100% methanol and stained with Hemacolor® (Merck KGaA, Darmstadt, Germany). Each blood smear was mounted with a cover glass using a mounting medium (O. Kindler GmbH, Freiburg, Germany) after confirming that they were dry.

All procedures for collecting samples from birds in this study were performed in accordance with the ethical standards of the Act on Welfare and Management of Animals 1973.

2.2. DNA Extraction and Molecular Detection of Avian Haemosporidia

DNA was extracted from the blood or tissue samples using either the standard phenol–chloroform method or QIAamp®DNA Micro Kit (QIAGEN, Hilden, Germany). Tris-EDTA buffer was used as the final buffer to dissolve the extracted DNA. DNA concentration was confirmed and adjusted to 50 ng/µL using Nanodrop One Microvolume UV-Vis Sprectrophotometers (Thermo Fisher Scientific, Waltham, MA, USA). Then, a nested polymerase chain reaction (PCR) targeting the partial mitochondrial cytochrome *b* (cyt*b*) gene of avian haemosporidia was carried out using a previously described protocol [29]. In brief, HaemNFI/HaemNR3 primer set was used for the 1st PCR. For the 2nd PCR, the HaemF/HaemR2 primer set was used for *Plasmodium* spp. and *Haemoproteus* spp., and the HaemFL/HaemR2L primer set was used for *Leucocytozoon* spp. [31]. The PCR products were then visualized using 1.5% agarose gels (Agarose S: Nippon Gene, Chiyoda, Japan) containing ethidium bromide (Nacalai tesque, Nakagyo, Japan). All obtained nucleotide sequences were compared with sequences in the GenBank database using the Basic Local Alignment Search Tool [32,33] and sequences in the MalAvi database [34].

2.3. Microscopic Detection and Infection Intensity of Avian Haemosporidia

Blood smears were examined under an Olympus BX43 of Olympus IX71 light microscope (Olympus, Tokyo, Japan). The smears were screened at 400× magnification and then carefully examined at 1000× magnification under oil immersion if a suspected creature was observed. Photos of the observed parasites were taken with cellSens Standard 1.6 (Olympus, Tokyo, Japan) and then morphologically classified into the proper genera [1]. Afterwards, intensity of infection (parasitemia) was estimated by counting the number of parasites per 10,000 erythrocytes. Counting of the erythrocytes was started at a random location, although repositioned in cases where there were overlapping erythrocytes. When an infected erythrocyte was found outside of the counted 10,000 erythrocytes, the parasitemia was calculated as 1/100,000 erythrocytes.

2.4. Statistical Analysis

To test whether infections were acquired over time, the initial and total parasite prevalence was compared using Fisher's exact test. The initial prevalence was calculated by the infection status of the first sample for each individual. The total prevalence was calculated by the infection status of all samples per individual. An individual was considered positive if one or more samples were positive by PCR and negative if all samples were negative by PCR. Among only individuals for which morphological detections were possible, the prevalence by PCR and by microscopy was compared using Fisher's exact test.

Parasite prevalence was compared with Fisher's exact test between environmental groups and vital status (live and deceased individuals). To reduce biases due to repeated sampling of particular individuals, only the first sample for each individual was used for

the above analysis. The parasitemia and prevalence of each parasite lineage were compared between vital status using Student's *t*-test and Fisher's exact test, respectively.

All statistical analyses were carried out with R version 4.3.2 [35] and were adjusted with Bonferroni correction. The 5% significance level was used to determine statistical significance throughout the study.

3. Results

3.1. Haemosporidian Prevalence by PCR

A total of 1966 samples from 1203 individuals of 12 species were collected. Collectively, 110 individuals of seven species in 18 prefectures were positive by PCR for either *Plasmodium* or *Haemoproteus* (Table 1, Supplementary Table S1). Note that one southern rockhopper penguin (*Eudyptes chrysocome*) was co-infected with *Plasmodium* and *Haemoproteus* parasites. *Leucocytozoon* was not detected in this study. Positive individuals were detected in all eight areas of Japan (Table S1). The total prevalence was 7.48% for *Plasmodium* and 1.75% for *Haemoproteus*. The initial prevalence using only the first sample of each individual was 5.40% and 1.08% for *Plasmodium* and *Haemoproteus*, respectively. There was no significant difference in prevalence between initial and total prevalence for *Haemoproteus* (Fisher's exact test: $p = 0.22$), even when compared among only live individuals of the outdoor group (Fisher's exact test: $p = 0.19$) and among only individuals that were sampled multiple times (Fisher's exact test: $p = 0.07$). Meanwhile, for *Plasmodium*, the total prevalence was significantly higher than the initial prevalence in all cases (Fisher's exact test: all samples $p = 0.05$; only live individuals of outdoor group $p = 0.03$; only multiple-sampled individuals $p < 0.01$).

Among individuals of the indoor group, three and one individual(s) were positive for *Plasmodium* and *Haemoproteus*, respectively. It is important to note that all four positive individuals had experienced relocation from a different facility where they were kept in outdoor enclosures. All other positive individuals were those of the outdoor group. The *Plasmodium* prevalence and odds of infection were significantly higher in outdoor individuals compared to indoor individuals (Fisher's exact test: OR = 6.37 $p < 0.01$), even when only live individuals were included (Fisher's exact test: OR = 4.93 $p < 0.01$; Figure 1). Meanwhile, although not statistically significant, the odds of infection were higher in outdoor individuals for *Haemoproteus* as well, both with and without deceased individuals (Fisher's exact test: all individuals OR = 3.52 $p = 0.32$, only live individuals OR = 3.42 $p = 0.31$; Figure 1).

By vital status, *Plasmodium* prevalence was 30.61% for deceased individuals and 5.32% for live individuals. The prevalence was significantly higher for deceased individuals compared to live individuals, even when compared among only outdoor individuals (Fisher's exact test: all individuals OR 9.95 $p < 0.01$, only outdoor individuals OR = 8.02 $p < 0.01$; Figure 1). Meanwhile, there was no significant difference between environmental groups for *Haemoproteus*, both with and without deceased individuals (Fisher's exact test: all individuals $p = 0.44$ odds ratio 1.86, only live individuals OR 1.55 $p = 0.50$; Figure 1).

Table 1. Summary of haemosporidian detection in penguins of this study, by species, environmental factor, and vital status.

Parasite Genus	Species [a]	Live						Deceased (All Outdoor)			Total		
		Indoor (Complete and Partial)			Outdoor								
		Individuals	Positive	Prevalence	Individuals	Positive [b]	Prevalence [b]	Individuals	Positive [b]	Prevalence [b]	Individuals	Positive [b]	Prevalence [b]
Plasmodium	King penguin (*Ap. patagonicus*)	36	0	0	18	0	0	3	0	0	57	0	0
	Emperor penguin (*Ap. forsteri*)	2	0	0							2	0	0
	Adelie penguin (*Py. adeliae*)	14	0	0							14	0	0
	Chinstrap penguin (*Py. antarcticus*)	15	0	0							15	0	0
	Gentoo penguin (*Py. papua*)	69	0	0	10	0 (1)	0 (10.0)				79	0 (1)	0 (1.27)
	Fairy penguin (*El. minor*)				2	0	0	10	0	0	12	0	0
	Humboldt penguin (*Sp. humboldti*)	21	0	0	574	27 (34)	4.70 (5.92)	23	12 (14)	52.17 (60.87)	618	39 (48)	6.31 (7.77)
	Magellanic penguin (*Sp. magellanicus*)	58	1	1.72	88	14 (19)	15.91 (21.59)	4	2	50.00	150	17 (22)	11.33 (14.67)
	African penguin (*Sp. demersus*)	24	1	4.17	167	4 (14)	2.40 (8.38)	7	0	0	198	5 (15)	2.52 (7.58)
	Macaroni penguin (*Es. chrysolophus*)	1	1	100							1	1	100
	N. rockhopper penguin (*Es. moseleyi*)	23	0	0	7	0	0				30	0	0
	S. rockhopper penguin (*Es. chrysocome*)	8	0	0	14	1	7.14	5	2	40.00	27	3	11.11
	total	271	3	1.11	880	46 (69)	5.23 (7.84)	52	16 (18)	30.77 (34.62)	1203	65 (90)	5.40 (7.48)
Haemoproteus	King penguin (*Ap. patagonicus*)	36	0	0	18	0	0	3	0	0	57	0	0
	Emperor penguin (*Ap. forsteri*)	2	0	0							2	0	0
	Adelie penguin (*Py. adeliae*)	14	0	0							14	0	0
	Chinstrap penguin (*Py. antarcticus*)	15	0	0							15	0	0
	Gentoo penguin (*Py. papua*)	69	0	0	10	1	10.00				79	1	1.27
	Fairy penguin (*El. minor*)				2	0	0	10	0	0	12	0	0
	Humboldt penguin (*Sp. humboldti*)	21	0	0	574	2 (5)		23	1	4.35	618	3 (6)	0.49 (0.97)
	Magellanic penguin (*Sp. magellanicus*)	58	0	0	88	4 (7)	4.55 (7.95)	4	0	0	150	4 (7)	2.67 (4.67)
	African penguin (*Sp. demersus*)	24	1	4.17	167	4 (5)	2.40 (3.00)	7	0	0	198	5 (6)	2.52 (3.03)
	Macaroni penguin (*Es. chrysolophus*)	1	0	0							1	0	0
	N. rockhopper penguin (*Es. moseleyi*)	23	0	0	7	0	0				30	0	0
	S. rockhopper penguin (*Es. chrysocome*)	8	0	0	14	0 (1)	0 (7.14)	5	0	0	27	0 (1)	0 (3.70)
	total	271	1	0.37	880	11 (19)	1.25 (2.16)	52	1	1.92	1203	13 (21)	1.08 (1.75)

[a] Genera are abbreviated: *Ap.* = *Aptenodytes*, *Py.* = *Pygoscelis*, *El.* = *Eudyptula*, *Sp.* = *Spheniscus*, *Es.* = *Eudyptes*. [b] Numbers in parentheses show the total number of positive individuals and prevalence (i.e., parasites detected at least once). Numbers in front of parentheses show the initial number of positive individuals and prevalence (i.e., infection status of only the first sample of each individual).

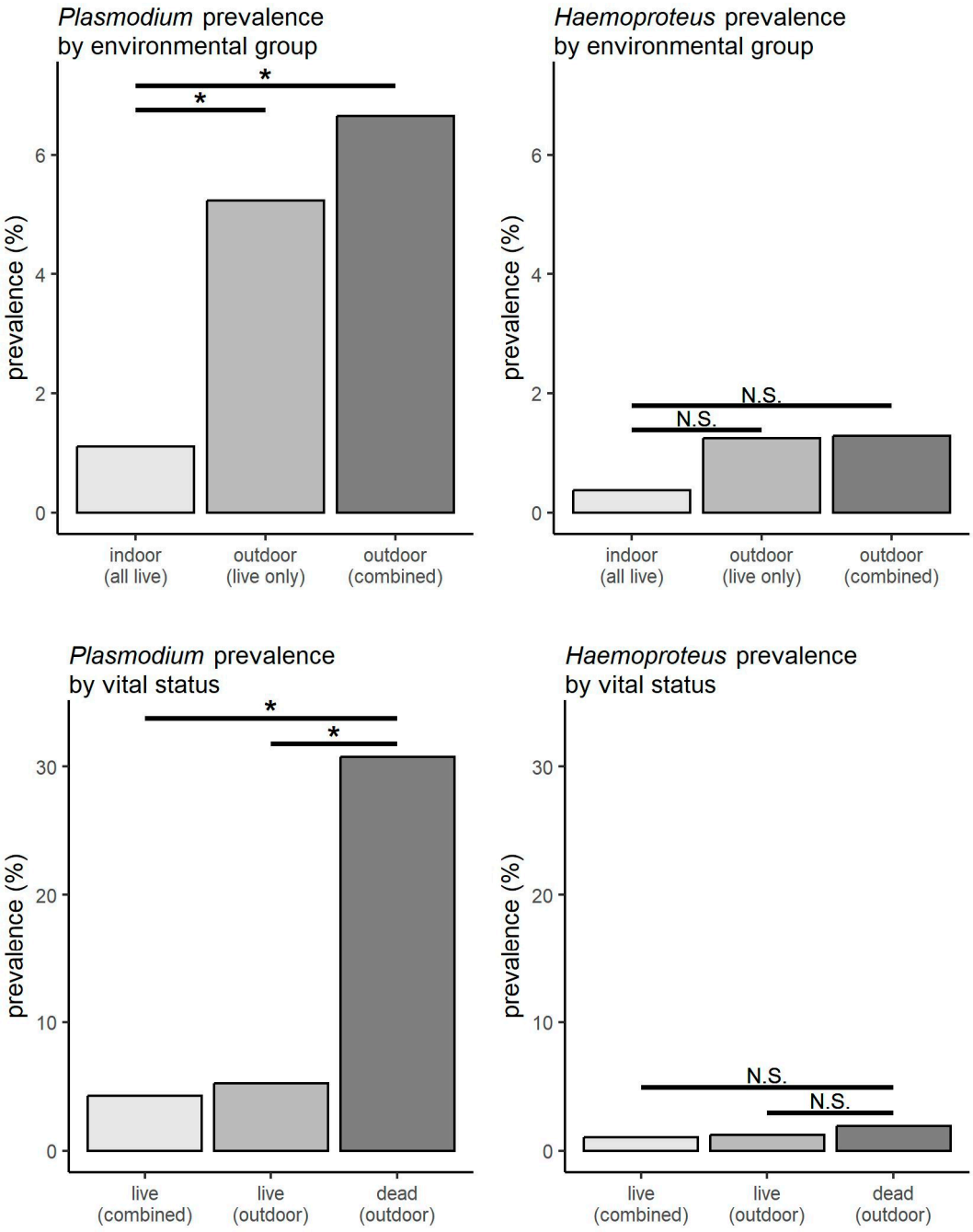

Figure 1. Parasite prevalences of penguins in this study, by environmental group and vital status. Asterisks (*) show significant differences ($p < 0.05$). N.S. = not significant.

3.2. Phylogeny

In total, 13 *Plasmodium* lineages and three *Haemoproteus* lineages were detected in this study (Table 2, Figure 2). Two *Haemoproteus* lineages named SPHUM06 and PYGPAP01 were added to the GenBank (accession nos. LC853581 and LC853582) and MalAvi database [34]. Lineages CXINA01, CXPIP10, GRW06, SPHUM03, SPHUM05, SYCON02, and TURPAL01 were detected from penguins for the first time in Japan (note that SPHUM03 and SPHUM05, which were reported in our previous studies, are included as part of this study). Combined with previously detected lineages, 27 parasite lineages were detected from penguins of Japan (Table 2).

Table 2. Summary of *Plasmodium* and *Haemoproteus* lineage that have been detected in penguins of Japan, based on MalAvi and GenBank.

Lineages [a]	Distribution		Species [b]	Detected Species in Japan [c]	Reference
pAPTPAT01	Japan only	host	1	***Ap. patagonicus* (HK, TY)**	MalAvi [d]
pCXINA01 *	Japan only	host	2	***Sp. humbodti* (TY, KU)**; ***Es. chrysocome* (TY)**	this study
		vector	1	*Cx. inatomii* (NI)	[36]
pCXPIP09 *	Japan only	host	11	*Larus argentatus* (CB); ***El. minor* (TY)**; ***Sp. humboldti* (HK, AK, TY, NI, KN, OS)**; ***Sp. magellanicus* (TY, NI, YN, HG, OI)**; ***Sp. demersus* (TY, KY, FO); *Es. chrysolophus* (TY); *Es. chrysocome* (NI)**; *Ardea cinirea* (NS); *Cyanopica cyanus* (TY); *Corvus corone* (CB); *Corvus macrorhynchos* (TY)	[19,29,37,38]; MalAvi; this study
		vector	5	*Cx. pipiens* (TY, KN); *Cx. inatomii* (NI); *Cx. sasai* (TY); *Lt. vorax* (KN)	[17,36,39,40]
pCXPIP10 *	Asia, Europe	host	2	***Sp. humboldti* (TY)**; *Botaurus sinensis* (CB)	[37]; this study
		vector	3	*Cx. pipiens* (NI); *Cx. inatomii* (NI)	[41]
pEUDCHR02	Japan only	host	1	***Es. chrysocome* (TY)**	MalAvi
pGALLUS02	Japan and Thailand	host	4	***Es. chrysocome* (NS)**; *Crossoptilon crossoptilon* (KN); *Streptopelia orientals* (KN)	[42]; MalAvi
		vector	2		
pGRW04 *	all except Antarctica	host	91	***Sp. humboldti* (TY, YA, KG)**; *Hypsipetes amaurotis* (TY); *Horornis diphone* (TY); *Zosterops japonicus* (TY); *Monticola solitarius* (TY)	[19,43]; MalAvi; this study
		vector	3	*Cx. pipiens* (TY, KN, NI); *Cx. quinquefasciatus* (OK)	[36,39,41,44]
pGRW06 *	all continents except Antarctica	host	103	***Sp. humboldti* (NS)**; ***Sp. magellanicus* (NI)**; *Hypsipetes amaurotis* (TY); *Horornis diphone* (TY); *Zosterops japonicus* (TY); *Troglodytes troglodytes* (ST); *Zoothera aurea* (TY); *Monticola solitarius* (TY); *Coccothraustes coccothraustes* (TY); *Chloris sinica* (TY)	[43,45]; this study
		vector	3		
pGRW11 *	Asia, Europe, Africa	host	55	***Sp. humboldti* (KN)**; ***Sp. magellanicus* (YA)**; ***Sp. demersus* (TY)**; ***Es. chrysolophus* (YA)**	[19]; MalAvi; this study
		vector	2	*Cx. pipiens* (TY); *Cx. quinquefasciatus* (OK)	[44,46]
pLINN1	Asia, Australia, Europe, North America	host	32	***Sp. humboldtii* (TY)**	MalAvi
		vector	6		
pLINOLI01	Asia, Europe, Africa	host	28	***Sp. demersus* (TY)**	MalAvi
pNYCNYC02 *	Japan only	host	5	***Sp. humboldti* (TY, KY, OS, OI)**; ***Sp. magellanicus* (IS, HG, OI)**; *Nycticorax nycticorax* (CB); *Luscinia cyanura* (ST); *Fringilla montifringilla*	[37,47]; MalAvi; this study
pPADOM02 *	Asia, Australia, Europe, Africa, North America	host	20	***El. minor* (TY)**; ***Sp. magellanicus* (OI)**; *Corvus corone*; *Passer montanus* (CB)	[37,48]; MalAvi; this study
		vector	6	*Ae. albopictus* (KN, NS); *Cx. bitaeniorhynchus* (NS); *Cx. inatomii* (NS); *Cx. pipiens* (TY, KN, NI); *Tr. bambusa* (KN); *Lt. vorax* (KN)	[17,29,38,39,46]
pSGS1 *	all continents except Antarctica	host	151	***Sp. humboldti* (TY, KN, KY, OS, YA, FO)**; ***Sp. magellanicus* (YN)**; ***Sp. demersus* (TY, KY)**; ***Es. chrysolophus* (TY, YA)**; *Hypsipetes amaurotis* (CB); *Spodiopsar cineraceus* (CB, KN)	[19,37]; MalAvi; this study
		vector	9	*Ae. albopictus* (KN); *Cx. pipiens* (TY, KN); *Cx. sasai* (TY); *Lt. vorax* (KN); *Cu. sigaensis* (KN)	[17,30,36,39,40,49]
pSPHUM01	Japan only	host	1	***Sp. humboldtii* (TY)**	MalAvi
pSPHUM02	Japan only	host	1	***Sp. humboldtii* (TY)**	MalAvi
pSPHUM03 *	Japan only	host	3	***Sp. humboldti* (NI, SZ)**; ***Sp. demersus* (SZ)**; *Calonectris leucomelas* (NI)	[29]; MalAvi; this study
pSPHUM05 *	Japan only	host	1	***Sp. humboldti* (HK, MG, NI)**	[29]; this study
		vector	1	*Cx. pipiens* (NI)	[29]

Table 2. *Cont.*

Lineages [a]	Distribution		Species [b]	Detected Species in Japan [c]	Reference
pSW5	Asia, Europe, Africa, North America	host	23	*Anas platyrhynchos* (HK, CB); *Fulica atra* (TY, CB); *Grus japonensis* (HK); *Podiceps cristatus* (CB); *Gallinago megala* (IB, CB, OK); *Gallinago hardwickii* (IB); ***Sp. magellanicus* (HG)**; *Calonectris leucomelas* (TY); *Botaurus eurhythmus* (NS)	[19,37,38,50,51]
pSYCON02 *	Japan and Spain	host	3	***Sp. humboldti* (KO)**; ***Sp. magellanicus* (OI)**	this study
		vector	1	*Cx. pipiens* (KN)	[39]
pTURPAL01 *	Japan only	host	2	***Py. papua* (HK)**; *Turdus pallidus* (OK)	MalAvi; this study
hAPPAT01	Japan only	host	1	***Ap. patagonicus* (HK)**	MalAvi
hHYPHI07	Asia	host	5	***Es. chrysocome* (HK)**; *Hypsipetes amaurotis* (CB); *Phylloscopus borealoides* (CB)	[37]; MalAvi
hPYGPAP01 *	Japan only	host	1	***Py. papua* (HK)**	this study
hSPHUM04	Japan only	host	1	***Sp. humboldtii* (KN)**	MalAvi
hSPHUM06 *	Japan only	host	1	***Sp. humboldtii* (SZ)**	this study
hSPMAG12 *	Japan only	host	5	*Larus crassirostris* (CB); ***Sp. humboldti* (TY, NI)**; ***Sp. magellanicus* (MG, IS, OI)**; ***Sp. demersus* (SZ, KY, OS)**; ***Es. chrysocome* (NI)**	[28,29]; MalAvi; this study

[a] Lineage names are according to the MalAvi database. The small case letter in front of the lineages represents the parasite genus (p = *Plasmodium*, h = *Haemoproteus*). Asterisks denote lineages detected in this study. [b] Number of species in which each lineage was detected, based on the MalAvi database (as of 14 January 2025) plus new host species found in this study. Lineages with no "vector" row have not been detected from any vector species. [c] Bold: penguin (Spheniscidae) species. Underline: detections from this study. Some genera are abbreviated: *Ap.* = *Aptenodytes*, *El.* = *Eudyptula*, *Sp.* = *Spheniscus*, *Es.* = *Eudyptes*, *Ae.* = *Aedes*, *Cx.* = *Culex*, *Tr.* = *Tripteroides*, *Lt.* = *Lutzia*. Parentheses denote the prefecture of detection: HK = Hokkaido, MG = Miyagi, IB = Ibaraki, ST = Saitama, CB = Chiba, TY = Tokyo, KN = Kanagawa, NI = Niigata, KY = Kyoto, HG = Hyogo, YA = Yamaguchi, FO = Fukuoka, NS = Nagasaki, OI = Oita, KG = Kagoshima, OK = Okinawa. [d] Unpublished records directly mined from MalAvi.

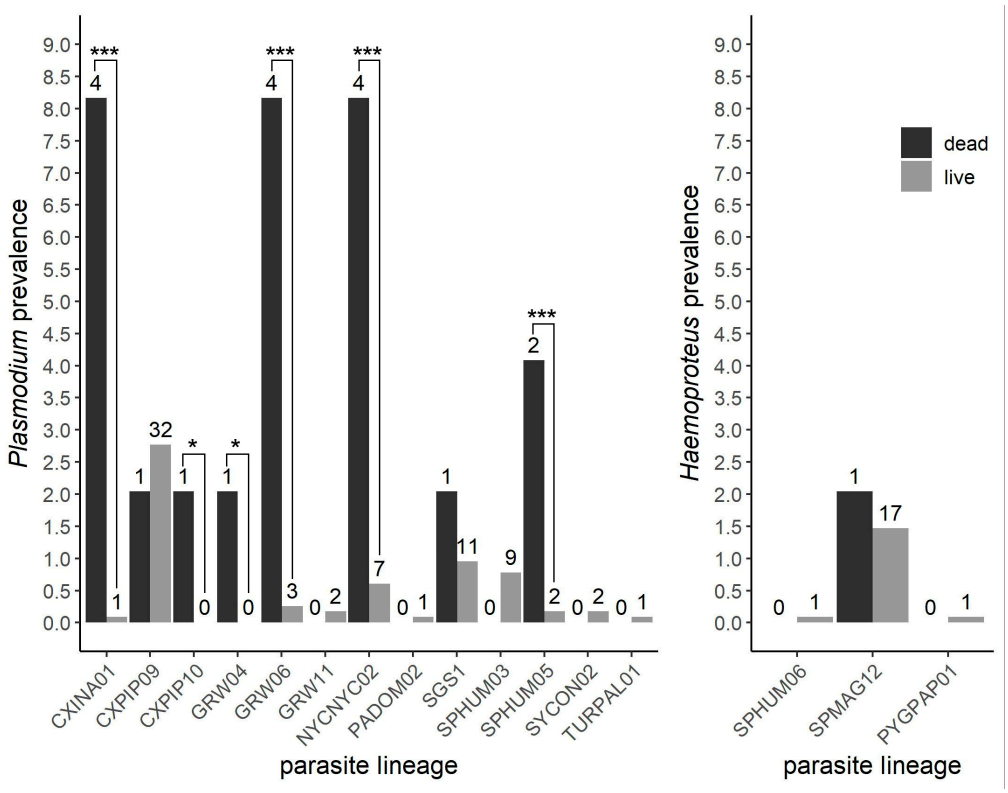

Figure 2. *Plasmodium* and *Haeamoproteus* prevalence by parasite lineage. Dark bars = dead individuals. Light bars = live individuals. Numbers above the bars are the number of positive individuals. Asterisks denote significant differences in prevalence between dead and live penguins (*: $0.01 \leq p < 0.05$, ***: $p < 0.01$).

In 6 of the 13 *Plasmodium* lineages, the odds of death were significantly higher among infected birds compared to non-infected birds (Fisher's exact test: CXINA01 OR = 94.25 $p < 0.01$, CXPIP10 $p = 0.04$, GRW04 $p = 0.04$, GRW06 OR = 31.53 $p < 0.01$, NYCNYC02 OR = 13.53 $p < 0.01$, SPHUM05 OR = 22.77 $p = 0.01$). In all other lineages, significant differences were not detected (SGS1 OR = 2.03 $p = 0.41$, SPMAG12 OR = 1.31 $p = 0.55$, all other lineages $p = 1$). Note that OR could not be calculated in some lineages because of zero values in one group.

3.3. Haemosporidian Prevalence by Microscopy and Parasitemia

By microscopy, the overall prevalence was 3.88% ($n = 37$) for *Plasmodium* parasites and 0.73% ($n = 7$) for *Haemoproteus* parasites (Table 3 and Table S2). Among individuals for which blood smears were prepared, the PCR prevalence was 5.67% (*Plasmodium*, $n = 54$) and 1.47% (*Haemoproteus*, $n = 14$), showing no significant difference in comparison to the prevalence by microscopy (Fisher's exact test: *Plasmodium* $p = 0.09$, *Haemoproteus* $p = 0.19$). One Magellanic penguin was positive for *Plasmodium* parasites by microscopy but negative by PCR. All other individuals positive by microscopy were also positive by PCR.

For individuals in which *Plasmodium* parasites were detected microscopically, the parasitemia was significantly higher in deceased individuals compared to live individuals (*t*-test: $p = 0.02$ t = −3.62 df = 4.02, GRW06 only $p = 0.05$, t = −3.07, df = 3.01; Figure 3).

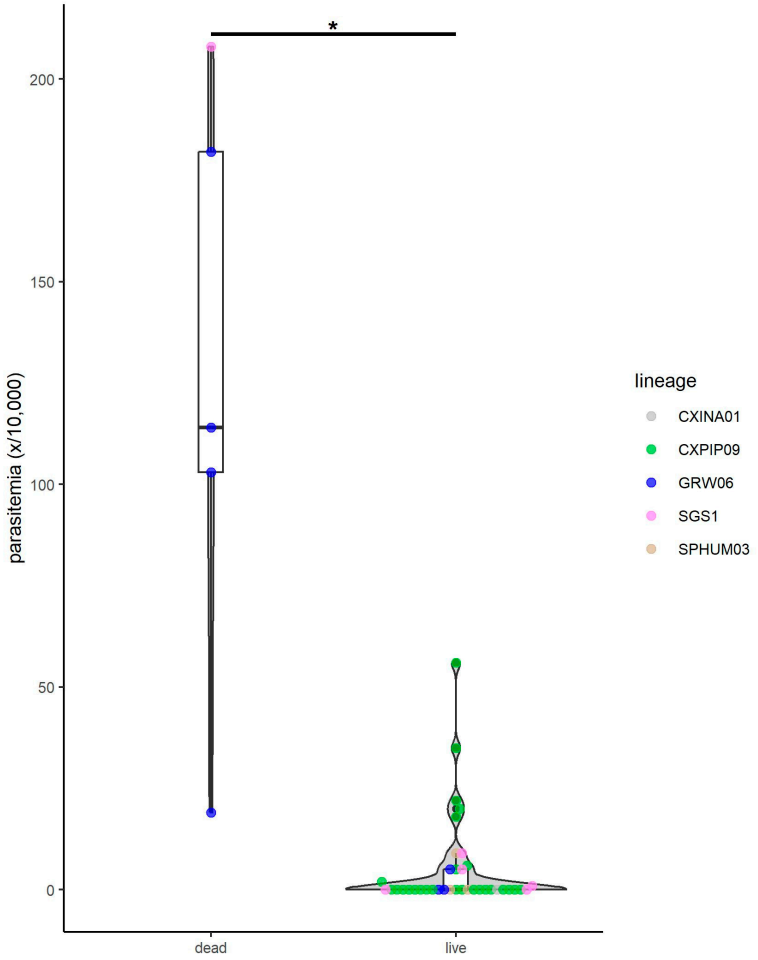

Figure 3. Parasitemia in *Plasmodium*-infected penguins, by vital status. Colors show different lineages, as shown in the legend. If an infected erythrocyte was found outside of the counted 10,000 erythrocytes, the parasitemia was calculated as 1/100,000 erythrocytes. The asterisk denotes a significant difference in parasitemia ($p < 0.05$).

Table 3. Summary of haemosporidian detection in penguins of this study in which blood smears were available.

Parasite Genus	Species [a]	Live				Deceased (All Outdoor)		Total	
		Indoor		Outdoor					
		Individuals	Positive [b]	Individuals	Positive [b]	Individuals	Positive [b]	Individuals	Positive [b]
Plasmodium	King penguin (*Ap. patagonicus*)	33	0/0	1	0/0			34	0/0
	Emperor penguin (*Ap. forsteri*)	2	0/0					2	0/0
	Adelie penguin (*Py. adeliae*)	14	0/0					14	0/0
	Chinstrap penguin (*Py. antarcticus*)	15	0/0					15	0/0
	Gentoo penguin (*Py. papua*)	61	0/0	1	0/0			62	0/0
	Fairy penguin (*El. minor*)			2	0/0			2	0/0
	Humboldt penguin (*Sp. humboldti*)	21	0/0	446	21/9	5	5/5	472	26/14
	Magellanic penguin (*Sp. magellanicus*)	58	1/1	84	14/10	1	0/0	143	15/11
	African penguin (*Sp. demersus*)	19	0/0	147	13/12	1	0/0	167	13/12
	N. rockhopper penguin (*Es. moseleyi*)	23	0/0	7	0/0			30	0/0
	S. rockhopper penguin (*Es. chrysocome*)	8	0/0	4	0/0			12	0/0
	total	254	1/1	692	48/31	7	5/5	953	54/37
Haemoproteus	King penguin (*Ap. patagonicus*)	33	0/0	1	0/0			34	0/0
	Emperor penguin (*Ap. forsteri*)	2	0/0					2	0/0
	Adelie penguin (*Py. adeliae*)	14	0/0					14	0/0
	Chinstrap penguin (*Py. antarcticus*)	15	0/0					15	0/0
	Gentoo penguin (*Py. papua*)	61	0/0	1	0/0			62	0/0
	Fairy penguin (*El. minor*)			2	0/0			2	0/0
	Humboldt penguin (*Sp. humboldti*)	21	0/0	446	1/0	5	0/0	472	1/0
	Magellanic penguin (*Sp. magellanicus*)	58	0/0	84	7/4	1	0/0	143	7/4
	African penguin (*Sp. demersus*)	19	1/0	147	4/2	1	0/0	167	5/2
	N. rockhopper penguin (*Es. moseleyi*)	23	0/0	7	0/0			30	0/0
	S. rockhopper penguin (*Es. chrysocome*)	8	0/0	4	1/1			12	1/1
		254	1/0	692	13/7	7	0/0	953	14/7

[a] Genera are abbreviated: *Ap.* = *Aptenodytes*, *Py.* = *Pygoscelis*, *El.* = *Eudyptula*, *Sp.* = *Spheniscus*, *Es.* = *Eudyptes*. [b] Left = PCR results; Right = microscopy results.

4. Discussion

While there have been sporadic reports of avian haemosporidia in Japan, only few facilities have been surveyed [19,27,29,52]. This is the first collective study to have surveyed avian haemosporidia in captive penguins throughout Japan. Parasites were detected from penguins in all eight areas of Japan, displaying the widespread distribution of avian haemosporidia amongst captive penguins. *Leucocytozoon* parasites were not detected in this study, possibly due to the relatively high specificity known in this genus or the lack of environments favored by vector blackflies [1,12,53]. Although information on relocation history was not available for many individuals, all except the two novel lineages have previously been detected from wild birds and/or vector insects of Japan, strongly suggesting that all individuals were infected in Japan.

The *Plasmodium* prevalence was significantly higher for the total prevalence compared to initial prevalence. Although not significant, eight initially negative individuals were found to be *Haemoproteus*-positive later on. As circannual fluctuations in parasitemia and medications may also be involved, it is difficult to confirm whether new infections were acquired [29]. Mosquitoes have been confirmed at zoological facilities [17,29,30]. While few investigations on biting midges have been carried out at zoological facilities in Japan, *Culicoides* biting midges have been confirmed at a facility in the Chubu area (pers. comm.). This suggests that new infections are possible at such zoological facilities, and along with the possibility of circannual fluctuations, it is important to perform periodical check-ups to investigate haemosporidian infection status.

As widely suggested, outdoor enclosures critically increase the exposure of vector insects to penguins and therefore the risk of avian malaria [18,54], as shown by data from this study. These results re-confirm that the ultimate and most effective prevention is to keep all penguins in indoor facilities. However, it is important to understand that not all facilities are capable of such environments, and other preventative measures such as medication and air curtains cannot be neglected.

Plasmodium infection can be highly lethal for penguins [18,54], as the odds of death were significantly higher for *Plasmodium*-infected individuals. Furthermore, the odds of death differed between parasite lineages, similar to previous studies in the US and UK [55,56]. Of the four lineages with particularly high odds of death (pCXINA01, pGRW06, pNYCNYC02, and pSPHUM05), all except GRW06 have only been detected in Japan and little information is known. GRW06 of *P. elongatum*, which is one of the most widespread lineages of *Plasmodium* spp., has been associated with mortality in penguins in New Zealand and Brazil [13,57,58]. This study adds evidence on the virulence of GRW06 in penguins. Furthermore, the *P. elongatum* parasitemia was significantly higher in deceased penguins, which was also seen in a previous study [57]. Although lethal cases with low parasitemia have also been reported [18], GRW06 might require high parasitemia in order to exhibit lethality. However, note that findings in this study may be a result of many factors such as sampling bias, medication, co-infections by other pathogens, and physiological factors [13,55,56].

Previous studies have suggested weak connections between *Haemoproteus* parasites and penguin mortality [12,59,60]. A previous study reports a lethal case in penguins due to haemoproteosis [27] but is considered problematic [28]. Meanwhile, gametocytes of SPMAG12 were detected from individuals including those that show no symptomatic signs, potentially related to the extremely low parasitemia [28]. The slightly high odds of death seen in this study may therefore be due to other factors rather than solely *Haemoproteus* infection.

There were several limitations upon data analysis of this study. Comparisons between penguin species were not possible, due to the strong bias between the number of individuals

and environments. Like in the UK [54], some of the most abundant species had the highest parasite prevalence. Furthermore, some species such as chinstrap penguins (*Pygoscelis antarcticus*) were kept exclusively indoors, while others such as Humboldt penguins tended to be kept outdoors, in relation to their natural environment. As found in this study, the captivity environment (indoors or outdoors) was a significant factor for haemosporidian prevalence, and the unbalanced numbers of each penguin species in each environment made analysis by species impossible. Additionally, information such as birth location, relocation history, medication history, and symptoms were not available for many individuals. Such information would make further analyses such as location of infection and virulence possible. Nonetheless, comprehensive studies such as this study are important to better understand the status of infection in penguins, which are crucial for preventative measures and ultimately the conservation of penguin populations. Furthermore, only roughly half of the facilities in Japan which keep penguins were investigated in this study. Future studies at uninvestigated facilities as well as continuous monitoring at already-investigated facilities are anticipated, both within Japan and throughout the world.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jzbg6010007/s1, Table S1: Summary of haemosporidian detection in penguins of this study, by prefecture, environmental factor, and vital status; Table S2: Summary of haemosporidian lineages detected from penguins in which blood smears were available; Figure S1: Map of prefectures investigated in this study.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Additionally, data on the detected parasite lineages have been deposited in the MalAvi database (http://130.235.244.92/Malavi/ (accessed on 14 January 2025)).

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