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Treatment of avian malaria in captive African penguins (*Spheniscus demersus*) by the combination of atovaquone and proguanil hydrochloride

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ABSTRACT

Avian malaria, a vector-borne disease caused by *Plasmodium* spp., poses significant threats to various bird populations, particularly captive penguins like the endangered African penguin (*Spheniscus demersus*). Penguins, originating from regions with low malaria prevalence, are highly susceptible when housed in malaria-permissive areas. This study evaluates the efficacy of an atovaquone/proguanil hydrochloride treatment protocol to manage avian malaria in a captive African penguin colony in an Italian zoo. The study involved 30 penguins monitored over 3 years. Thirteen penguins tested positive for *Plasmodium* spp., with 11 undergoing treatment. The treatment protocol consisted of atovaquone/proguanil hydrochloride (10/4 mg/kg) administered orally for 3 days, repeated after a week. Post-treatment monitoring at 7, 30, and 60 days, and follow-ups up to 2 years, showed that all but one penguin cleared the infection. The treatment was well tolerated, with no adverse effects observed. The findings suggest that this protocol is effective as a treatment of avian malaria and could be a valuable tool in avian malaria management, particularly for endangered species in captivity. However, the persistence of *Plasmodium relictum* in one case highlights the need for careful post-treatment monitoring to prevent recurrence or reinfection. The study underscores the importance of developing tailored antimalarial protocols for captive birds to enhance conservation efforts and mitigate the risks posed by avian malaria.

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1. Introduction

Avian malaria is a vector-borne disease caused by hemoparasites belonging to the genus *Plasmodium* [1]. To date, more than 50 *Plasmodium* species and 1,500 lineages have been identified to infect birds, with *Plasmodium relictum*, *Plasmodium matutinum,* and *Plasmodium elongatum* being the most commonly identified [2,3]. The parasite is transmitted by mosquitoes of the *Culex* genus [4]. The parasite life cycle consists of asexual exo-erythrocytic, erythrocytic and post-erythrocytic stages in the host, where a subpopulation of parasites produces micro- and macrogametocytes that can be ingested by mosquitos [4,5]. Gametogenesis, zygote formation, and oocyst formation occur within the mosquito, which can transmit the latter to a new host, replicating the cycle [5].

Avian malaria-related plasmodia (AMRP) have been described to infect birds across 23 orders in every continent except Antarctica [1,6]. However, the susceptibility to the disease may vary significantly depending on the host species, individual immunity, and parasite lineage [7–9]. Among the avian species, penguins (Order: Sphenisciformes) are particularly vulnerable to avian malaria, and they are widely regarded as one of the most affected groups in both wild and captive settings [10–13]. Although definite evidence is lacking, penguins are considered naïve species [14] likely because their natural habitats, characterized by cold, dry, or windy conditions, have low prevalence, or absence, of malaria parasites and their vectors [12,15,16]. Consequently, captive penguins hosted in avian malaria-permissive regions are especially prone to the disease [6,14]. The course of the disease can be acute or chronic, with clinical signs ranging from absent to severe. Common manifestations include weight loss, respiratory distress, lethargy, weakness, general depression, vomiting, and enteric disorders [6], while critical cases may present neurological signs [1]. Mortality rate remains a subject of debate [14]. Recently, some authors observed that avian malaria can compromise the overall health of captive penguin colonies, contributing to increased co-morbidity and mortality [17].

Outbreaks of symptomatic avian malaria in captive penguin populations may severely affect endangered

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species, for which conservation is a primary objective of zoological gardens and rehabilitation centres. Among those, *Spheniscus demersus*, native to Southern Africa, has experienced a significant population decline in recent decades, insomuch that it has been classified as endangered on the IUCN red list [18]. Conservation guidelines for maintaining these birds in captivity underscore the importance of disease prevention, early diagnosis and treatment [19]. However, specific prophylactic and therapeutic protocols against AMRP are lacking. Over the years, several treatments have been proposed. Some reports describe the treatments with pyrimethamine, usually combined with sulphonamides [14], both inhibitors of folate synthesis in protozoa [20]. However, teratogenicity and associated folic acid depletion in hosts make them unsuitable for prolonged treatments [21]. Other drugs, such as doxycycline, chloroquine, primaquine, and mefloquine, have been used off-label [14,22]. These drugs are unregistered for use on wild animals and administered off-label with variable efficacy [23], posing challenges for developing standardized therapeutic protocols [24] and raising concerns about toxicity [25].

More recently, the combination of atovaquone and proguanil, also unregistered for veterinary use, has shown promise in wild birds. Atovaquone, a hydroxynaphthoquinone, inhibits the mitochondrial electron transport of the parasites at the cytochrome bc_1 complex, while proguanil hydrochloride is a biguanide that selectively inhibits folate biosynthesis. Together, those compounds exhibit schizonticidal activity in blood and tissues in humans [26]. Additionally, they have been reported to inactivate gametocytes and inhibit the oocyst development in vectors [14,27]. Widely used to treat human malaria, providing good results in terms of efficacy and safety [26], this combination has been proposed for treating haemosporidian infections in buzzards, greenfinches and other bird species [27,28]. However, its application in penguins has been limited, with protocols often extrapolated from human treatments and employing high doses [29]. Recently, a tailored atovaquone/proguanil hydrochloride protocol demonstrated efficacy and safety in snowy owls (*Bubo scandiacus*) against *P. relictum* [30].

In the light of those findings, this study evaluates the efficacy of a low-dose treatment with atovaquone/proguanil hydrochloride for managing avian malaria infections in a colony of captive African penguins (*S. demersus*) hosted in an Italian zoo.

2. Materials and methods

2.1. Colony description

The colony of African penguins was housed at the Giardino Zoologico di Pistoia (Tuscany, Italy). The zoo is situated in a peri-urban area with abundant vegetation, and it spans 7 ha. It accommodates almost 1,300 specimens representing 91 animal species. As a Full Member of the European Association of Zoos and Aquaria, the institution adheres to strict regulations and high standards for animal welfare and enforces conservation actions.

The penguin exhibit encompasses an outdoor area of approximately 1,050 m^2, equipped with a pool suitably sized for swimming, and small shelters, mainly hollow rocks, used as nesting and breeding sites. This environment allows potential contact with local wildlife and exposure to flying arthropods, including potential vectors. Comprehensive hygienic measures and good management practices are implemented to minimize mosquito circulation and larval proliferation. The zoo also housed, in separate and isolated exhibits, other bird species that have been associated with *Plasmodium* spp. infections in the MalAvi database [31]. Those species include *Phalacrocorax carbo*, *Phoenicopterus roseus*, *Struthio camelus*, *Tyto alba,* and *Bubo bubo*.

The study was carried out between September 2021 and March 2024. At the outset, the colony consisted of 30 birds (17 males and 13 females) aged between 4 months and 25 years. Most birds were born in the zoo, and the others were imported from another European zoological garden. Between 2021 and 2023, five chicks were born; however, two (one male and one female) died at 4 months due to a hyperacute form avian malaria, confirmed through post-mortem findings and molecular detection of *P. relictum* from organs, including the liver. An adult male penguin with no previous known infection by hemoparasites died of unknown causes in 2022, and a female died in December 2023 due to aspergillosis. Post-mortem testing revealed *P. relictum* in the latter but not in the former.

Antimalarial prophylaxis with pyrimethamine was routinely administered to all penguins each year, from March–April to October–November, depending on the presence of mosquitos, which in some years extended to March and November. The prophylactic regimen consisted of 12.5 mg per bird, orally administered every third day, following established protocols [24].

2.2. Monitoring and sampling

Hemoparasite monitoring commenced in October 2020, after the post-mortem diagnosis of avian malaria in a pullet (identified as ZNZ) without clinical signs before death. Screenings were carried out every 6 months, specifically in spring and autumn, immediately before and after the period of mosquito circulation, respectively. Parasite monitoring occurred before and 15 days after prophylaxis to ensure sufficient drug clearance. Juveniles under 5 months were

excluded from the study to avoid disrupting parental care.

Blood samples (<1 mL) were collected by expert veterinary personnel from the coccygeal vein and immediately placed in tubes with heparin, sent via refrigerated overnight shipment to the Department of Veterinary Medicine of the University of Bari. Only one blood sample was collected from each penguin, except for those testing positive and treated, which underwent treatment and follow-up sampling.

2.3. Biomolecular investigation, sequencing and sequence analysis

Immediately upon arrival, total genomic DNA was extracted from 30 µL of whole blood by using the ZymoBIOMICS® DNA Miniprep (Zymo Research Corporation, Irvine, USA) following the manufacturer's instructions.

Samples underwent nested PCR (nPCR) targeting the partial mitochondrial cytochrome b (*cytb*) gene of avian haemosporidians as previously described [32,33]. Amplicons were analysed on 1.5% agarose gel and visualized under UV light after staining with 0.5 µg/mL ethidium bromide.

Amplicons were purified using the PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (ThermoFisher Scientific, Milan, Italy) and sequenced at the Microsynth Seqlab facilities (Göttingen, Germany) using the primers for the nPCR 2nd step HAEMN-F (5"-ATGGTGCTTTMGATATATGCATG-3') and HAE MN-R2 (5'- GCATTATCTGGATGTGATAATG GT-3") [33].

Nucleotide sequence analysis was performed with CLC Genomic Workbench v. 22.0.1 (Qiagen Digital Insight, Aarhus, Denmark). Contigs were aligned using CAP3 [34], and primer sequences were removed from the final assembly.

In one case, chromatograms showed double peaks, indicating coinfection. Therefore, the PCR product was cloned into pTZ57RT using the InsTAclone PCR Cloning Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Chemically competent *Escherichia coli* TOP10 cells (ThermoFisher Scientific) were used for transformation. Twelve randomly selected clones were sequenced, and sequences were assembled and compared among themselves using the ClustalW algorithm implemented in CLC Genomics Workbench. ClustalW was also employed to compare the nucleotide sequences obtained before and after the treatments.

All sequences were compared with those in GenBank for identification through BLAST software

Table 1. Detection of *Plasmodium* spp. from African penguins treated with atovaquone/proguanil hydrochloride during the survey period.

Penguin code	Sex	Age	Treatment start	D0	GenBank accession number	D7	D30	D60	M12	M18	M24
ZNZ	Female	Pullet	Not appliable	*Plasmodium relictum* PreBa1679	na	np	np	np	np	np	np
SKP	Male	Pullet	Not appliable	*Plasmodium matutinum* PmaBa1948	na	np	np	np	np	np	np
DRT	Male	Adult	17/11/2021	*Plasmodium* sp. Psp1Ba1729	PQ197113	(–)	(–)	(–)	(–)	ns	(–)
BHI	Female	Adult	17/11/2021	*Plasmodium matutinum* PmaBa1733	PQ197114	(–)	(–)	(–)	(–)	ns	(–)
UGO	Male	Adult	11/03/2022	*Plasmodium relictum* PreBa1870	PQ197115	(–)	(–)	(–)	(–)	(–)	ns
NTL	Female	Adult	11/03/2022	*Plasmodium relictum* PreBa1872	PQ197116	(–)	(–)	(–)	(–)	np	np
ALX	Male	Adult	11/03/2022	*Plasmodium relictum* PreBa1874a *Plasmodium* sp. Psp2Ba1874b	PQ197117 PQ197118	(–)	(–)	(–)	(–)	(–)	ns
VND	Female	Adult	11/03/2022	*Plasmodium relictum* PreBa1875	PQ197119	(–)	(–)	(–)	(–)	(–)	ns
HPE	Female	Adult	11/03/2022	*Plasmodium* sp. Psp1Ba1878	PQ197120	*Psp*	(–)	(–)	(–)	(–)	ns
RMN	Male	Adult	28/11/2022	*Plasmodium* sp. Psp3ba2062	PQ197121	(–)	(–)	(–)	(–)	ns	ns
DMS	Male	Adult	30/03/2023	*Plasmodium relictum* PreBa2252	PQ197122	*Pre*	*Pre*	*Pre*	ns	ns	ns
PRT	Male	Adult	30/03/2023	*Plasmodium* sp. Psp1Ba2255	PQ197123	*Psp*	(–)	(–)	ns	ns	ns
KEV	Male	Youngster	21/07/2023	*Plasmodium relictum* PreBa2562	PQ197124	*Pre*	(–)	(–)	ns	ns	ns

D7: 7 days after treatment; D30: 30 days after treatment; D60: 60 days after treatment; M12: 12 months after treatment; M18: 18 months after treatment; M24: 24 months after treatments; *Pre*: *Plasmodium relictum*; *Psp*: *Plasmodium* sp.; (-): negative for *Plasmodium* spp.; na: not available; np: not performed because of the death of the bird; ns: not scheduled. D7, D30, and D60 are referred to the post-treatment monitoring; M12, M18, and M24 are referred to the routine monitoring.

[35] with the default settings and submitted to GenBank under accession numbers PQ197113 – PQ197124 (Table 1).

2.4. Treatment and follow-ups

Out of the 13 penguins testing positive for *Plasmodium* spp., 11 were included in the study and treated within a few days after the pathogen detection. The same protocol was applied to all the infected birds with follow-up monitoring conducted at consistent intervals. Treatment dates are indicated in Table 1. The pullets ZNZ and SKP were not includedas Plasmodia were detected post-mortem.

The treatment protocol comprised atovaquone/proguanil hydrochloride at 10/4 mg/kg, administered orally via fish feed once daily for 3 days. The cycle was repeated 7 days later [30]. Treatment completion was designated as Day 0 (D0), while the beginning of treatment is indicated as D-13. Blood samples were collected on Day 7 (D7), 30 (D30), and 60 (D60) post-treatment and analysed by nPCR to assess the infection status. Additional follow-up occurred at 12 months (M12) and, for some birds, at 18 or 24 months (M18 and M24). Details are provided in Table 1.

2.5. Haematological investigations

Blood samples collected from the treated penguins at D-13 and during follow-up were analysed for key markers of hepatic and renal functions. Specifically, haematic albumin (ALB), total proteins (TP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB), and creatinine (CRE) levels were measured. Complete biochemical profiles were available only for seven penguins, and only these individuals were included in the marker analysis. The normal distribution of data was confirmed by grouping the parameters from all penguins using the Shapiro–Wilk test, and one-way repeated measure ANOVA was employed to assess the potential time–response relation for each parameter, considering that the observations within each penguin were correlated. Pairwise comparisons between time points were carried out by applying the pairwise *t*-test with Bonferroni correction. The significance threshold was set at $p < 0.05$. All the statistical analyses were performed in R v. 4.4.2 [36], with the package rstatix [37].

3. Results

No *Haemoproteus* spp. were detected, while *Plasmodium* spp. DNA was amplified from 13 African penguins, including the two pullets that died of malaria before blood screening could be carried out. Consequently, 11 penguins were treated with atovaquone/proguanil hydrochloride. None of them exhibited signs of avian malaria. Details on hemoparasite identification are provided in Table 1. After sequence assembly and primer trimming, the length of the nucleotide sequences of the amplicons was 478 bp.

One penguin (code ALX) was coinfected with *P. relictum* and another undescribed *Plasmodium* sp. (accession numbers PQ197117 and PQ197118, respectively). *Plasmodium relictum* was detected in five other penguins (namely, UGO, NTL, VND, DMS, and KEV), *P. matutinum* in one (BHI) while the other four penguins (DRT, HPE, RMN, and PRT) were found infected by yet undefined *Plasmodium* spp (Table 1).

Seven days after treatment (D7), hemoparasites were not detected in the blood of seven penguins, while four others remained infected. In those cases, the nucleotide sequences of the amplicons were 100% identical to those obtained at the time of diagnosis before treatment.

By D30, all but one treated penguin tested negative for hemoparasites, and they remained negative at D60 and in subsequent follow-ups. The first African penguins to be treated (DRT and BHI) tested negative for up to 2 years after treatments, while those treated in March 2023 were found negative for up to 18 months. A female penguin (NTL) that was included in the study and tested negative since D7 died from aspergillosis approximately 17 months after treatment.

In one case, a treated penguin (code DMS) remained positive for *P. relictum* at D7, D30 and D60. Further follow-up was not scheduled as it fell outside the study temporal scope. The nucleotide sequence of the amplicon obtained before treatment (named PreBa2252 and submitted in GenBank with the accession number PQ197122) was 100% identical to sequences obtained from the same animal after treatment. It was also 100% identical to pre-treatment sequences from penguins NTL, ALX, VND and KEV, corresponding to the expected portion of the mitochondrial *cytb* gene of *P. relictum*.

Throughout and after treatment, all animals remained in good health, with no adverse events observed by zookeepers or veterinary staff. Hepatic and renal markers evidenced no significant increase in their haematic concentrations (Table 2). A significant increase in the average ALB concentration was observed at D30 compared to D13 ($p = 0.006$), but levels returned comparable to D13 on D60. Despite wide individual variability, no significant differences were observed in specific hepatic (ALT and AST) or renal (TB and CRE) markers. The penguin NTL showed no signs of disease, malaise, or other adverse conditions till about 1 month before death. Biochemical markers at follow-up were not

Table 2. Average values of the biochemical markers for hepatic and renal function before treatment (D13) and seven (D7), 30 (D30), and 60 (D60) days after treatment. The *F* and *p* values of the repeated measure ANOVA statistics after sphericity corrections are reported. Values that significantly differ between themselves in the pairwise comparison have been reported with the same superscript letter.

	Albumin (g/dL)	Total proteins (g/dL)	Alkaline Phosphatase (U/L)	Alanine Aminotransferase (U/L)	Aspartate aminotransferase (U/L)	Total Bilirubin (mg/dL)	Creatinine (mg/dL)
D-13	2.31 ± 0.29^{a}	5.82 ± 1.30	225.17 ± 136.25	155.17 ± 115.70	137.00 ± 29.78	1.76 ± 0.65	0.42 ± 0.08
D7	2.54 ± 0.12	5.05 ± 0.96	193.00 ± 97.53	101.00 ± 46.18	137.00 ± 55.00	1.80 ± 0.99	0.39 ± 0.08
D30	$2.77 \pm 0.28^{a,b}$	5.79 ± 1.18	285.43 ± 160.17	127.86 ± 37.28	121.40 ± 27.57	1.56 ± 0.30	0.48 ± 0.12
D60	2.36 ± 0.21^{b}	4.76 ± 1.20	158.00 ± 75.22	190.86 ± 102.89	175.57 ± 86.30	1.27 ± 0.26	0.35 ± 0.05
F	12.13	1.379	2.643	1.525	0.912	0.901	3.786
p	<0.001	0.287	0.106	0.256	0.446	0.443	0.043

significantly altered. Despite *P. relictum* being detected in the liver post-mortem, no significant signs of avian malaria were observed during the necropsy. The death was unequivocally attributed to severe aspergillosis. Data were not enough to establish or exclude a relationship between *P. relictum* and aspergillosis, and further investigations are in progress.

4. Discussion

Avian malaria poses a significant threat to the wild and captive bird populations [14], particularly when naïve hosts are exposed to parasites and vectors with which they have not coevolved, leading to ineffective defences against the disease [5,16,38].

This issue is exacerbated by climate change, which expands the range of potential mosquito vectors of AMRP [39,40] and increases their geographical distribution [6]. Other than naïve wild birds, avian malaria jeopardizes captive birds, and penguins hosted in zoos, aquatic parks or rehabilitation centres are highly exposed to the disease. During the last few years, several reports have highlighted the wide circulation of AMRP and its deleterious impact on penguin colonies [6,29], two of them in Italy [17]. This may impair the zoo efforts for the conservation of endangered species, such as, for instance, *Spheniscus mendiculus* (Galapagos penguin), *S. demersus* (African penguin), *Spheniscus humboldti* (Humboldt penguin), which are classified as endangered or vulnerable by IUCN, with decreasing population trends [41]. Among those, *S. demersus* is also classified as largely depleted [42].

Consequently, the availability of safe and effective treatment protocols is critical for the management of the disease in captive colonies.

In this study, atovaquone/proguanil hydrochloride therapy demonstrated high efficacy, as out of the 11 treated penguins, all but one turned negative for hemoparasites within 30 days. The treatment was effective against different AMRP species, including *P. relictum*, *P. matutinum* and three lineages of still undefined species. Some birds tested positive on D7, but they were negative by D30, likely due to the prolonged activity of the drugs. Atovaquone and proguanil hydrochloride lipophilic properties and high plasma protein binding (up to 75% and 99% for atovaquone and proguanil, respectively) [26] contribute to their persistence in the bloodstream. In humans, atovaquone has a half-life ranging from 2 [43] to 5.9 days [44], providing prophylactic activity for at least 1 week after administration of a single dose [45]. Based on this pharmacokinetic profile, the therapeutic protocol (i.e. 2 three-day cycles with a seven-day interval) was designed to maintain effective drug concentrations for at least 4 weeks, assuming that pharmacokinetic features in penguins could be comparable to humans. Although no pharmacodynamic (i.e. EC_{50}) and pharmacokinetic data for atovaquone and proguanil in animals are available [27], this protocol sensibly reduced the number of required administrations compared to other regimens, which often involve 14 [29] to 21 [28] doses. The current protocol consisted of six administrations of atovaquone/proguanil hydrochloride 10/4 mg/kg per day, effectively clearing AMRP in all but one penguin, and this reduction is significant for captive management where ensuring the drug intake is challenging.

The single treatment failure observed in the penguin DMS was evident immediately after the protocol ended, suggesting the insusceptibility of the parasite rather than reinfection or relapse. Resistance to atovaquone has been extensively documented in human malaria-related plasmodia [46]. Notably, the emergence of resistant human malaria-related plasmodia has sometimes been observed during the therapy following the initial apparent clearance of the parasite [47]. The inappropriate or excessive use of antimalarial drugs is a well-recognized driver of resistant strain selection [48], and this concern extends to atovaquone/proguanil hydrochloride. However, recent studies suggest that prophylactic use in humans does not appear to promote the selection of resistant *P. falciparum* strains [49]. Resistance to atovaquone is often driven by point mutations in the mitochondrial gene *cytb* [46], but mutations associated with resistance were shown to impair parasite fitness significantly [49]. The partial nucleotide sequences of *cytb* from the plasmodia in this study did not include

mutations considered associated with resistance. Therefore, the resistance of the *P. relictum* infecting the penguin DMS, if associated with *cytb*, would be due to mutation in the unsequenced region. To mitigate the risk of resistance selection, treatment was limited to individuals with confirmed *Plasmodium* spp. infection.

Importantly, no adverse events were observed during or after treatment. Biochemical analyses showed no significant changes in hepatic or renal markers, confirming the treatment's safety.

Although one treated penguin (NTL) died of aspergillosis 17 months after the treatment, the long interval and the negative test results prior to death exclude a direct relationship with the therapy. The detection of *P. relictum* in the liver at necropsy suggests either reinfection or a residual infection. The presence of wild birds and other species potentially susceptible to AMRP in the zoo may represent a reservoir for reinfection, thus increasing the risk for penguins to be infected. Still, the probability of such occurrences is very low, considering that the other species are well separated from penguins. A further possibility is that wild birds could act as reservoirs of AMRP [50]. Unfortunately, no data about the circulation of AMRP in wild birds from that area are available, so their contribution remains hypothetical despite being a solid possibility. Conversely, the possibility of relapse cannot be entirely excluded. The potential existence of dormant stages, analogous to hypnozoites in human malaria [51], remains unconfirmed in AMRPs [52]. Considering that atovaquone and proguanil act by interfering with the mitochondrial electron transport and the pyrimidine biosynthesis of the parasite, respectively [53], they are active against both hepatic and blood stages [45] but not against quiescent cells. While data from this study and others suggest long-lasting negative results post-treatment, further research is needed to confirm these findings. Apart from its direct therapeutic effects, reducing parasite circulation may lower the overall infective pressure within colonies [29]. Nevertheless, limitations of blood-based diagnostics, such as the impossibility to detect dormant stages, highlight the need for robust post-treatment monitoring.

Recently, some studies highlighted the positive correlation between stress levels in hosts and *P. relictum* replication rate [54] and the possibility of coinfection of other pathogens with AMRP [55,56]. This study did not collect data on stress hormones, and no coinfections were observed, except for the aspergillosis of NTL, but further investigations may explore those possibilities.

In conclusion, the atovaquone/proguanil hydrochloride protocol (10/4 mg/kg per day, for 2 three-day cycles with one-week interval) proved to be safe and effective in managing avian malaria in a captive *S. demersus* colony. This combination expands the available options for avian malaria control in zoological and rehabilitation settings, which can be particularly menaced by *Plasmodium* spp. circulation. However, careful post-treatment monitoring is essential to detect possible therapeutic failures or reoccurrences and to mitigate the risk of resistance. Prophylactic use of this protocol should be avoided to preserve its efficacy and prevent the potential selection of resistant strains.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The nucleotide sequences of the diagnostic PCR products have been submitted in GenBank with the accession numbers PQ197113-PQ197124

Ethical approval

All procedures, diagnostic investigations and treatments that involved animals were approved by the Ethical Committee of the Department of Veterinary Medicine of the University of Bari (Italy) with approval number 22/2022 and in compliance with the current European and Italian regulations.

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