

## ORIGINAL ARTICLE

# Treatment with chloroquine is retinotoxic in captive African penguins (*Spheniscus demersus*). Attenuation and recovery of electroretinographic responses

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## Abstract

**Purpose:** To evaluate the effect of prophylactic anti-malarial chloroquine treatment, and its cessation, on electroretinographic (ERG) responses of captive African penguins.

**Methods:** A brief ERG protocol (“QuickRetCheck”) was recorded under mesopic conditions with manual restraint and no sedation or pupil dilation. Birds were recorded on two separate occasions, first while being treated with a daily chloroquine dose of 10 mg/kg for 12 days ( $n = 15$ , treatment group) and second after 4 months without chloroquine treatment ( $n = 6$ , off-treatment group). Three birds were recorded on both occasions. Three other birds from the flock that died were studied histopathologically.

**Results:** Scotopic responses were unmeasurable in either recording and therefore were not analyzed. Mean a- and b-wave amplitudes of the mixed rod-cone responses to standard ( $3 \text{ cd}\cdot\text{s}/\text{m}^2$ ) and high ( $10 \text{ cd}\cdot\text{s}/\text{m}^2$ ) intensity flashes were higher in the off-treatment group. No difference in implicit times was observed. Sex, age, and number of previous chloroquine treatments did not affect ERG responses. Histopathology revealed *Plasmodium* spp. in the lungs, liver, and brain, but not in the eyes, of the necropsied birds, and there were no signs of retinitis or retinopathy.

**Conclusions:** Daily chloroquine treatment was associated with attenuated ERG responses in penguins, which improve following cessation of treatment. Further work is warranted to establish a chloroquine dose that is efficacious yet has minimal adverse effects. Our results suggest that ERG responses of captive penguins undergoing ERG for any indication (such as prior to cataract surgery), must be evaluated in light of the birds' anti-malaria treatment status.

## KEYWORDS

avian malaria, chloroquine, ERG, penguin, *Plasmodium*, retinal toxicity

## 1 | INTRODUCTION

Chloroquine (CQ) and its less toxic analogue, hydroxychloroquine (HCQ), were originally developed as anti-malarial

drugs in the middle of the previous century.<sup>1</sup> Since then, several immunomodulatory properties of these drugs have been discovered and the indications for CQ and HCQ treatment in humans are rapidly expanding. These include

treatment of autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis, as well as other dermatological and oncological conditions.<sup>2,3</sup> Most recently, pre-clinical and clinical studies have been launched to evaluate the safety and efficacy of CQ and HCQ in the treatment of the novel coronavirus SARS-COV-2 causing the current COVID-19 pandemic.<sup>4,5</sup> The use of CQ in veterinary medicine is not as common and is mostly restricted to treatment of avian malaria,<sup>6</sup> although a recent publication suggests the use of CQ in treating cats suffering from feline infectious peritonitis.<sup>7</sup>

Chloroquine and HCQ are considered safe and well tolerated, and are on the World Health Organization's list of essential medicines, which includes the most efficacious, safe, and cost-effective drugs for priority conditions.<sup>8</sup> However, the use of CQ and HCQ has potential adverse effects, and these are broadly divided into two categories. The first includes gastrointestinal and cutaneous manifestations that are rather frequent and usually disappear with dose reduction. The second category includes rare but more severe signs of retinal toxicity, and less commonly cardiac abnormalities, that are associated with long-term treatment and higher doses of the drugs.<sup>2</sup> In humans, a daily HCQ dose greater than 5 mg/kg for over 10 years is associated with a higher risk of retinal toxicity.<sup>9</sup> Consequently, the American Academy of Ophthalmology recommends a maximal daily CQ dose of 2.3 mg/kg.<sup>10</sup>

Retinal toxicity was originally considered to be a very rare side effect of CQ treatment that occurs in less than 1% of human patients, but recent studies that assessed only patients receiving long-term treatment with high doses show that the prevalence is as high as 7.5%.<sup>9</sup> The classic sign of retinal toxicity associated with CQ and HCQ treatment in humans is a parafoveal ring of depigmentation termed "Bull's eye maculopathy". However, manifestations of retinal toxicity can be demonstrated using automated visual fields, spectral domain optical coherence tomography (SD-OCT) and electroretinography (ERG) well before the appearance of this ophthalmoscopic sign, and even before the patient has any visual deficits such as blurring of vision and letters dropping from words.<sup>2</sup> SD-OCT findings in human patients suffering from HCQ/CQ induced retinal toxicity include decreased foveal and parafoveal thickness and disruption of the ellipsoid zone around the fovea. In mild and moderate cases, treatment cessation results in stabilization of the retinopathy without loss of visual acuity; however, cases with severe retinopathy at the time of diagnosis show progressive damage even after the drug is discontinued.<sup>11</sup> Full-field and multifocal ERG (mfERG) responses are attenuated in human patients suffering from HCQ/CQ induced retinal toxicity. Cessation of treatment may lead to improvement of ERG responses if the diagnosis is made in early stages; however, as with SD-OCT, severe cases fail to show improvement in ERG responses when the treatment is discontinued.<sup>12</sup>

In the current study, we investigated the effect of CQ treatment on ERG responses of captive African penguins (*Spheniscus demersus*) in the Tisch Family Zoological Gardens in Jerusalem, Israel. Penguins are highly susceptible to avian malaria, a mosquito-borne disease caused by the protozoan parasite *Plasmodium*.<sup>6</sup> Clinical signs of malaria in penguins are non-specific and include loss of appetite, weight loss, respiratory signs, lethargy, pale mucus membranes, green feces, isolation from the group and vomiting.<sup>6,13</sup> However, during outbreaks it is common to find dead birds that did not present any antemortem clinical signs of the disease. The disease is seasonal; in the Northern Hemisphere morbidity and mortality occur mostly during spring-summer months, due to the high prevalence of mosquitoes at this time of the year.<sup>6,13</sup>

*Plasmodium* infection has been documented in wild penguin populations, but outbreaks of avian malaria are more frequent and severe in captive penguin populations. A recent survey in Northern Hemisphere zoos revealed that avian malaria was diagnosed in 12.5% of penguin flocks, and the incidence of infection in African penguins in rehabilitation centers in South America and South Africa was 17%–34%.<sup>6</sup> Therefore, prophylaxis is extremely important in controlling infection rates in captive penguins. Prophylactic measures are practiced in zoos worldwide and include eliminating mosquitoes, creating barriers between mosquitoes and birds, and the use of drug prophylaxis.<sup>6</sup>

Avian malaria in the penguin colony of the Tisch Family Zoological Gardens in Jerusalem is a major concern; among 30 birds that died in the zoo between 2014 and 2020, 13 were positive for *Plasmodium* spp. on Giemsa stained blood smears and/or PCR. In order to minimize morbidity and mortality, penguins in the zoo are prophylactically treated with a weekly dose of CQ between March and November of every year, and with a daily dose during clinical outbreaks. In November 2018, the entire colony was transferred to a temporary habitat due to renovations of the penguin exhibition. Since stress is suspected to exacerbate *Plasmodium* morbidity,<sup>14</sup> penguins received a daily dose of CQ for 12 days prior to the transfer. This provided us with the opportunity to assess retinal function in birds being treated with CQ using full-field ERG. Retinal function of treated birds was compared to that of birds recorded in February 2020, while the colony was not receiving any CQ treatment.

## 2 | MATERIALS AND METHODS

### 2.1 | Husbandry

The African penguin colony of the Tisch Family Zoological Gardens in Jerusalem consists of 38 birds, including breeding pairs. Birds are housed in a habitat measuring 400 m<sup>2</sup> consisting of a rocky terrain and a large freshwater pool. Glass walls

and a fine meshed mosquito net enclose the habitat. The birds are hand-fed fish three times daily and receive avian vitamin supplements (Mazuri<sup>®</sup>, small birds supplement with vitamin A, 5M25) four times a week.

## 2.2 | Chloroquine treatment regimen

The zoo's penguins are prophylactically treated with oral chloroquine phosphate (Resochin<sup>®</sup>; Bayer Pharmaceuticals) between March and November ("mosquito season") of every year at a dose of 10 mg/kg once a week. Prior to an anticipated stressful event, or when clinical signs of disease are observed, the frequency of treatment increases and the same dose is administered daily for 10–14 days.

## 2.3 | Study animals

The study was conducted in accordance with the guidelines of the Association for Research in Vision and Ophthalmology and approved by the zoo's Institutional Animal Care & Use Committee. Eighteen penguins were studied, including nine males, eight females, and one bird of unknown gender.

In November 2018 we recorded ERGs from 15 randomly selected birds that had been treated with CQ to minimize morbidity due to the stress of moving to a temporary enclosure ("treatment group"). Birds were treated orally with a daily dose of 10 mg/kg for 12 consecutive days prior to the recording. The mean  $\pm$  SE (median, minimum – maximum) age of the 15 birds was  $6.8 \pm 1.4$  (5.5, 0.8 – 19.0) years. Following recording, the anterior segment was evaluated, and pupillary light reflex tested, with a slit lamp (Kowa SL-17). To minimize handling time, this examination was performed only in 8/15 birds.

In February 2020 we recorded ERGs from six randomly selected birds (three of which were also recorded in November 2018) that had not been treated with CQ for approximately 4 months prior to the recording ("off-treatment group"). The mean  $\pm$  SE (median, minimum – maximum) age of the six birds was  $9.6 \pm 2.6$  (8.6, 1 – 20.2) years. To minimize handling time, no ophthalmic examination was performed.

## 2.4 | Electroretinography

Unilateral recordings of a randomly chosen eye were conducted under mesopic conditions, in a darkened indoor facility. To minimize the amount of time that the birds were out of their enclosure, and lessen the resulting stress, animals were not dark-adapted. No chemical restraint was used. Instead, animals were manually restrained by an experienced keeper and positioned in lateral recumbency. To improve conduction, the recorded eye was kept moist with a drop of 1.4% hydroxymethylcellulose.

Signals were recorded using a gold loop electrode that was placed under the clear nictitating membrane to ensure direct contact with the cornea. Subcutaneous needles (CareFusion) served as reference and ground electrodes and were placed at the ipsilateral lateral canthus and the back of the neck, respectively. Impedance was kept under 5 K $\Omega$ . Pupils were not dilated as the iris muscles in penguins are mostly striated and are unresponsive to parasympatholytic mydriatic drugs.<sup>15</sup> The only publication we have found reporting pharmacological mydriasis in penguins involved intracameral injection of a muscle relaxant,<sup>16</sup> which was not justifiable for the purpose of this study. While other protocols for achieving mydriasis using muscle relaxants in various avian species have been published, some require application of multiple drops at 15 min intervals<sup>16,17</sup>; dangerous side effects have been reported<sup>17</sup> and their use would have required repeated capturing of the birds and unacceptable stress. This may explain why ERGs have previously been recorded in 79 free-living raptors with manual restraint and without pupil dilation, just as we did in the present study<sup>18</sup>.

All recordings were conducted using a Handheld Multi-species Electroretinography system (HM<sub>s</sub>ERG, OcuScience, Henderson, NV) with a bandpass of 0.3–300 Hz. Rod and mixed rod-cone responses were recorded using the HM<sub>s</sub>ERG's pre-programmed "QuickRetCheck" protocol. This brief protocol was introduced in 2010 by Labelle et al<sup>19</sup> who recorded ERGs in kangaroos, and because of its short duration it "allows for quick analysis of retinal function in exotic species." Responses to four flashes, presented at 0.5 Hz and a light intensity of 0.01 cd-s/m<sup>2</sup>, were recorded and averaged to generate a single scotopic flash response. Mixed rod-cone function was recorded in response to one standard flash and one high-intensity flash (3 and 10 cd-s/m<sup>2</sup>, respectively). A 50 Hz notch filter was used in the analysis of the responses, to reduce ambient electric noise.

## 2.5 | Pathology and histopathology

Three birds from the flock died in late November—early December, 2018, within 3 weeks of cessation of CQ treatment and the first ERG recording. They included a 5.5-year-old male and two females, aged 1.3 and 3.8 years. None of these birds underwent ERG recording prior to its death. The eyes were rapidly enucleated starting with a circumferential incision at the fornix, continuing with dissection of the extraocular muscles, and ending with severing the optic nerve. The remnants of periocular skeletal muscle, fat, and connective tissue were gently trimmed to expose the sclera and visualize the posterior ciliary artery. The globes were submerged in 90 ml of Davidson's solution (Glacial acetic acid, 10 ml; 95% ethyl alcohol, 30 ml; 10% neutral buffered formalin, 20 ml; Distilled water, 30 ml) for 48 h. Globes were then placed in 70% ethanol solution for 24 h, transferred to 90% ethanol

solution for 24 more hours and finally transferred to 100% ethanol solution for 24 h. Prior to cutting, globes were placed in a bone decalcification solution (10% EDTA/TRIS-HCl) for 24 h. The fixed and decalcified globes were then cut open by an incision made from back to front, perpendicular to the posterior ciliary artery, starting adjacent to the optic nerve and ending with the cornea. The trimmed specimens were placed in a cassette and underwent standard histologic preparation. Sections 4  $\mu\text{m}$  thick were cut and stained with H&E.

Following gross necropsy, specimens from the liver, lungs, brain, heart, kidney, intestine, stomachs, spleen, and striated muscles were fixed in a 10% buffered formalin solution for 48 h, followed by standard histologic preparation.

## 2.6 | Signal and statistical analysis

Scotopic responses to the 0.01  $\text{cd}\cdot\text{s}/\text{m}^2$  flash were unmeasurable in any of the recordings and therefore were not analyzed. A- and b-wave amplitudes of the standard- and high-intensity mixed rod-cone responses were measured from baseline to the first trough and from that trough to the most positive peak following that trough, respectively. When the pre-stimulus signal was “noisy”, an average value was calculated and served as baseline. Implicit times (IT), which are the respective time intervals between the stimulus onset to the trough or to the positive peak, were measured to examine the response kinetics.

Statistical analysis was conducted using JMP<sup>®</sup> Pro 15.0.0 (SAS institute Inc., 2016) and GRAPHPAD Prism version 5.0. The Shapiro-Wilk test was used to confirm normal distribution of the data. As all birds are prophylactically treated with CQ every year, and during clinical outbreaks and stressful events, the age of the birds is highly correlated with, and reflects, the number of previous courses of CQ treatment. The effect of age and number of previous courses of CQ treatment on ERG parameters was assessed by correlation analysis. Repeated measures ANOVA was used to assess the effects of treatment and sex on a- and b-wave amplitudes and IT of the three birds that were recorded both with and without CQ treatment. Two-way ANOVA was used to assess the effects of treatment and sex on a- and b-wave amplitudes and IT for the other 15 birds in the treatment and off-treatment groups. *p*-Values of .05 or lower were considered statistically significant.

## 3 | RESULTS

### 3.1 | Clinical findings

No abnormalities were detected in 5/8 treated birds that underwent an evaluation of the anterior segment in Nov 2018. Three treated birds presented with varying degrees of

sub-epithelial corneal opacities compatible with dystrophy. Two birds had diffuse central opacities (~4 mm diameter) and the third had a linear opacity spanning 3 mm from the central cornea temporally.

### 3.2 | Electroretinography

As the penguins were not dark-adapted, and their pupils not dilated, the 0.01  $\text{cd}\cdot\text{s}/\text{m}^2$  flashes did not yield a measurable signal in any of the birds and therefore the single flash scotopic response was not included in the analysis. The standard-intensity mixed rod-cone responses to the 3  $\text{cd}\cdot\text{s}/\text{m}^2$  flashes were measurable in 11/15 and in 6/6 birds in the treatment and off-treatment groups, respectively. The high-intensity mixed rod-cone responses to the 10  $\text{cd}\cdot\text{s}/\text{m}^2$  flashes were measurable in 9/15 and 5/6 birds in the treatment and off-treatment groups, respectively. Mean ERG responses of treatment and off-treatment groups to the standard- and high-intensity stimuli are presented in Table 1, and the recorded and averaged traces are presented in Figure 1. Using correlation analysis, no significant correlation was found between bird age \ number of previous CQ treatments on the one hand and a- and b- wave amplitudes of the standard and high-intensity mixed rod-cone responses on the other hand (Spearman's  $\rho = .16$  and .06 for the standard-intensity a- and b-wave amplitudes respectively and Spearman's  $\rho = .40$  and .07 for the high-intensity a- and b-wave amplitudes respectively).

Repeated measures ANOVA was used to assess the effects of treatment and sex on the measured responses in the three birds that were recorded on both occasions. Their mean a- and b-wave amplitudes in response to the standard-intensity flash (3  $\text{cd}\cdot\text{s}/\text{m}^2$ ) were significantly higher while being off-treatment. A similar trend was observed in response to the high-intensity flash (10  $\text{cd}\cdot\text{s}/\text{m}^2$ ), although statistical significance was not achieved. Figure 2 presents the responses to both the 3- and 10  $\text{cd}\cdot\text{s}/\text{m}^2$  stimuli in a single bird that was recorded in both sessions.

Two-way ANOVA was used to assess the effects of treatment and sex on ERG parameters in the remaining 15 birds. A-wave amplitudes of the off-treatment group were significantly higher than those of the treatment group in response to both flash intensities, with a similar trend that did not reach statistical significance observed for b-wave amplitudes. No difference was observed in ITs of the responses, and the sex of the bird had no effect on ERG responses, in any of the analyses (Table 1).

### 3.3 | Pathology and histopathology

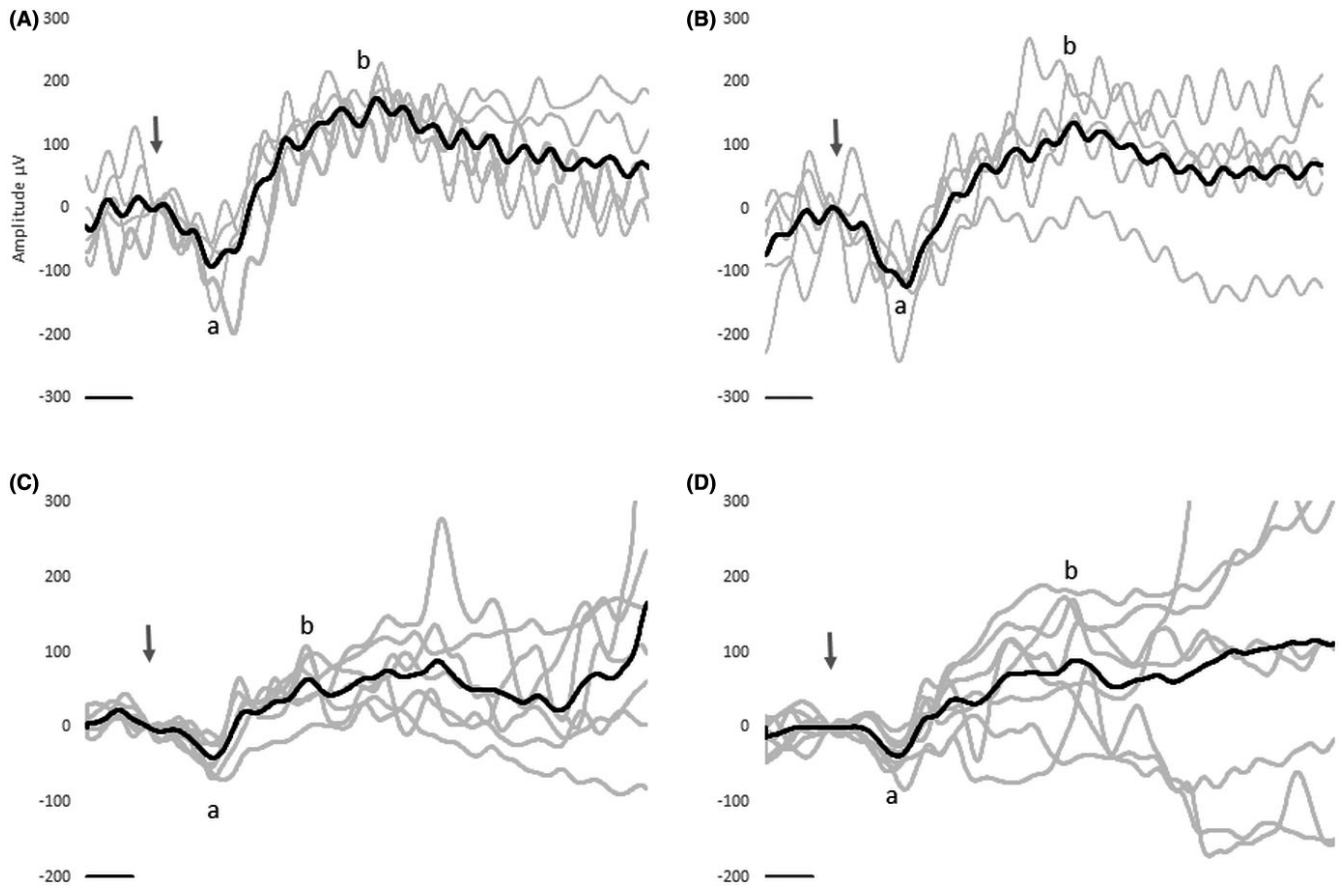
All carcasses, organs and histopathology slides were examined by a Diplomate of the American College of

TABLE 1 ERG responses of alert African penguins with and without treatment with Chloroquine phosphate

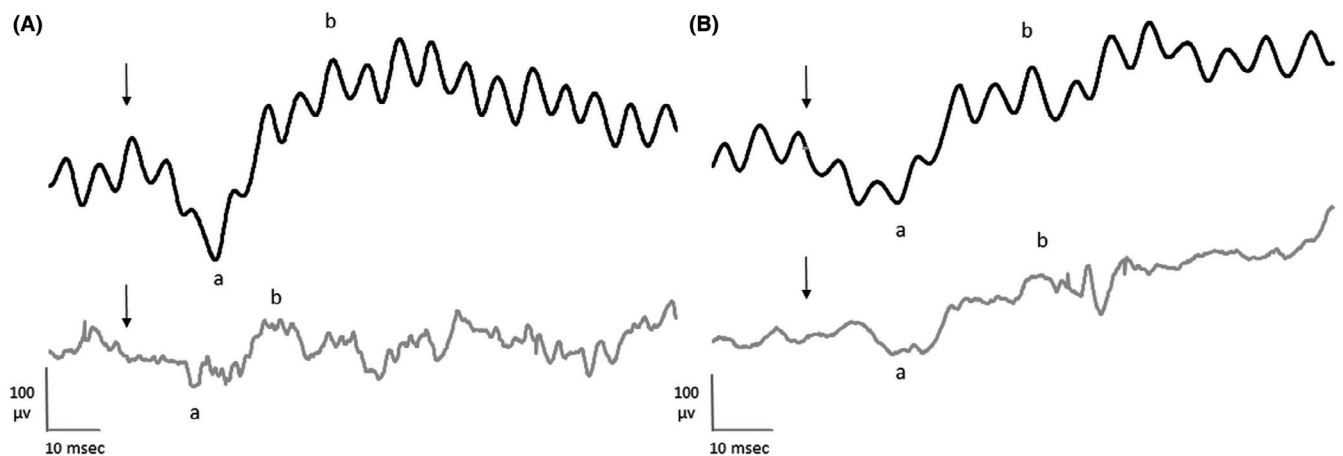
	Repeated birds			Non-repeated birds		
	Treated (N = 3)	Off-treatment (N = 3)	p-Value effect of treatment	Treated	Off-treatment	p-Value effect of treatment
3 cd-s/m <sup>2</sup> a-wave amplitude (μV)	51.7 ± 18.9	221.9 ± 37.4	.007*	59.7 ± 5.4 N = 8	102.1 ± 9.2 N = 3	.003*
3 cd-s/m <sup>2</sup> a-wave IT (ms)	15.6 ± 1.7	14.6 ± 1.9	.85	14.7 ± 0.5 N = 8	12.5 ± 0.6 N = 3	.08
3 cd-s/m <sup>2</sup> b-wave amplitude (μV)	180.8 ± 45.4	416.5 ± 32.1	.009*	239.0 ± 49.7 N = 8	334.1 ± 14.4 N = 3	.38
3 cd-s/m <sup>2</sup> b-wave IT (ms)	47.0 ± 6.6	59.6 ± 5.9	.42	47.5 ± 3.9 N = 8	47.7 ± 2.4 N = 3	.83
10 cd-s/m <sup>2</sup> a-wave amplitude (μV)	61.1 ± 20.1	185.3 ± 23.9	.17	62.0 ± 6.6 N = 6	146.4 ± 8.3 N = 2	.002*
10 cd-s/m <sup>2</sup> a-wave IT (ms)	13.8 ± 0.3	13.9 ± 1.4	.78	14.3 ± 0.5 N = 6	13.3 ± 1.5 N = 2	.09
10 cd-s/m <sup>2</sup> b-wave amplitude (μV)	144.1 ± 30.4	391.1 ± 112	.09	181.8 ± 27.5 N = 6	308.7 ± 0.9 N = 2	.28
10 cd-s/m <sup>2</sup> b-wave IT (ms)	38.4 ± 7.4	51.1 ± 4.3	.51	47.61 ± 3.6 N = 6	49.7 ± 2.6 N = 2	.56

Note: Results are presented as mean ± SE. Repeated measures ANOVA was used for analysis of three birds recorded twice (Repeated birds) and two-way ANOVA was used for analysis of the remaining 15 birds (Non-repeated birds). p-Values for the effect of treatment, and for the effect of sex are presented and \* indicates a significant difference between the treatment and off-treatment groups.

Abbreviations: ERG, electroretinographic; IT, implicit time of the response; N, numbers of eyes with measurable responses averaged for each parameter.



**FIGURE 1** Electoretinographic (ERG) traces of penguins from CQ treatment and off-treatment groups recorded with the “QuickRetCheck” protocol under mesopic conditions. In each panel, traces from all birds with measurable responses, and the averaged trace, are shown in gray and black, respectively. (A) Standard-intensity mixed rod-cone response to the 3 cd·s/m<sup>2</sup> flashes of the off-treatment group ( $n = 6$ ). (B) High-intensity mixed rod-cone response to the 10 cd·s/m<sup>2</sup> flashes of the off-treatment group ( $n = 5$ ). (C) Standard-intensity mixed rod-cone response of birds treated with chloroquine 10 mg/kg SID for 12 days ( $n = 11$ ). (D) High-intensity mixed rod-cone response of birds treated with chloroquine 10 mg/kg SID for 12 days ( $n = 8$ ). Flash onset is indicated by an arrow; a- and b-wave peaks are marked with “a” and “b”; horizontal bars represent 10 ms



**FIGURE 2** Electoretinographic (ERG) responses of the same bird recorded with ((lower trace, gray)) and without treatment ((upper trace, black)) with chloroquine at a dose of 10 mg/kg SID for 12 days. (A) Response to a single 3 cd·s/m<sup>2</sup> stimulus. (B) Response to a single 10 cd·s/m<sup>2</sup> stimulus. Flash onset is indicated by an arrow; a- and b-wave peaks are marked with “a” and “b”

Veterinary Pathologists. No gross or histopathological lesions were seen in any of the eyes. No signs of retinopathy, retinitis, retinal pigment epithelium (RPE) damage or other disease were seen in any of the retinas or pectens (Figure 3).

Histopathological lesions were observed in the lungs, liver and brain of all birds (data not shown). In the lungs there was a histiocytic interstitial pneumonia with multiple intracytoplasmic parasitic schizonts (measuring up to 10  $\mu\text{m}$ ); schizonts contained up to twenty round basophilic merozoites, each 1–2  $\mu\text{m}$  in diameter. Macrophages occasionally contained intracytoplasmic granular to spicular, brown to black, variably birefringent pigment (hemozoin). Lymphohistiocytic hepatitis with similar parasites was seen in all livers. Similar parasites were seen in the brains, but with minimal inflammatory infiltrate. The parasites were identified as *Plasmodium* spp. (Avian malaria). The morphologic diagnosis was interstitial, histiocytic, diffuse moderate pneumonia, with intrahistiocytic and intraerythrocytic schizonts, and intrahistiocytic hemozoin, the etiology consistent with avian malaria (*Plasmodium* spp.).

## 4 | DISCUSSION

In the current study, captive African penguins treated with CQ for the prevention of avian malaria showed lower a- and b-wave amplitudes of mixed rod-cone ERG responses, compared to the off-treatment group of penguins from the same colony. A comparable effect of CQ on full-field ERG responses was shown in rats<sup>20</sup> and similar findings were described in human patients suffering from severe HCQ retinal toxicity, with lower ERG amplitudes,<sup>21,22</sup> particularly of the mixed rod-cone responses.<sup>23</sup>

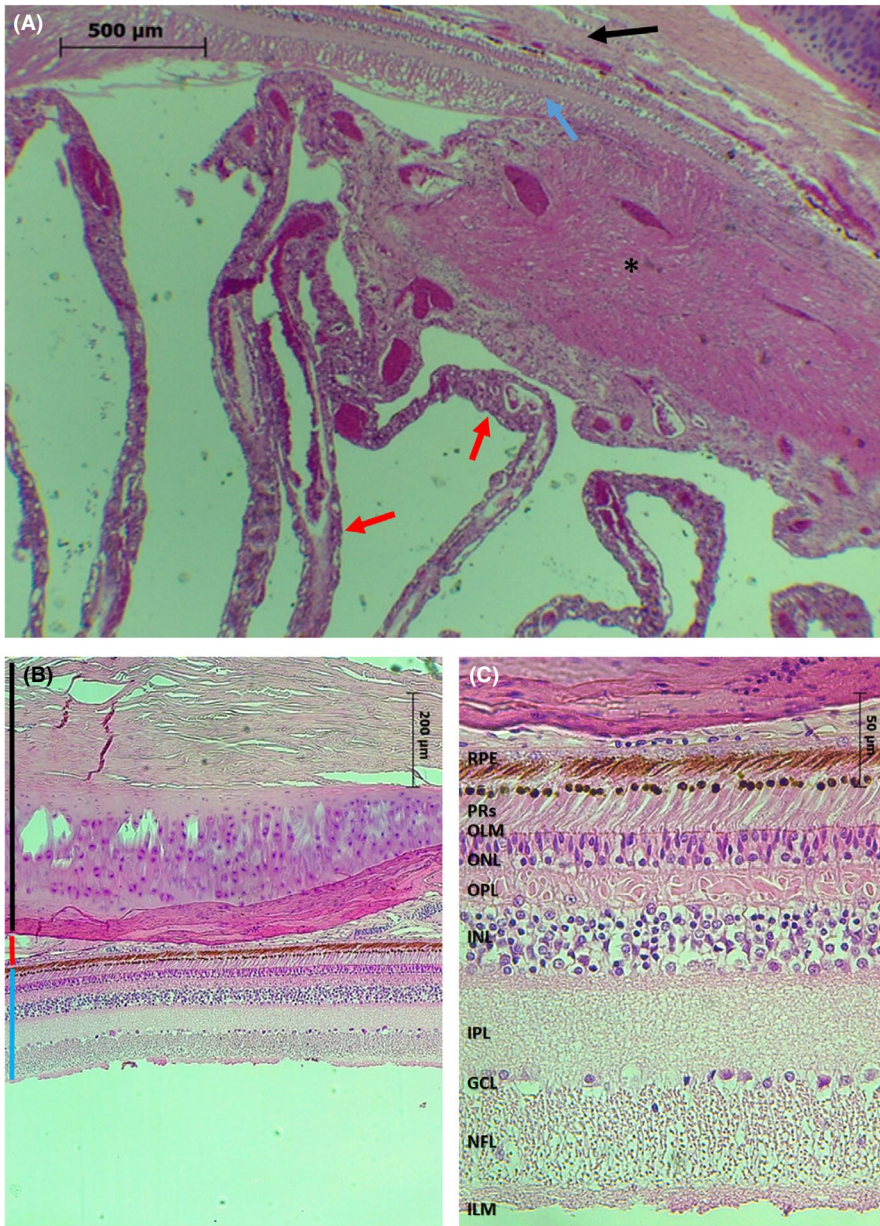
Analysis of the responses of the three birds that were recorded while being treated and off-treatment suggests that the effect of CQ on the penguin ERG, though considerable, might be reversible. This is because the recordings performed during treatment (characterized by low amplitudes) preceded the off-treatment recordings (characterized by significantly higher amplitudes) of the same birds. Moreover, no correlation was found between the number of previous treatments per bird and a- and b- wave amplitudes, nor did we see any histopathological retinal changes, all suggesting lack of cumulative damage to the retina. Retinal toxicity of CQ and HCQ in humans may likewise be reversible in some cases after cessation of the treatment,<sup>24,25</sup> but there are several reports of progression of the pathology despite cessation of the medication, mainly seen as enlargement of areas of fundus autofluorescence and thinning of retinal layers on SD-OCT.<sup>10,26–28</sup> In a recent study Marmor *et al* report that cases with RPE involvement at the time of treatment cessation show progression of retinal damage on SD-OCT,

including foveal thinning and loss of cone structure, while cases in which medication was ceased before RPE damage occurs regain normal structure.<sup>11</sup> Since we found no evidence of RPE involvement in any of the birds studied histopathologically, this could explain the reversible nature of the ERG deficits suggested by our data. Admittedly, none of the eyes we studied electroretinographically were available for histopathology. However, in six eyes of three birds from the same flock, who were treated with an identical protocol just weeks prior to their death, as well as in previous years, we did not see any histopathological retinal lesions (Figure 3). This suggests that the ERG deficits we saw could be reversible because CQ treatment did not cause long-lasting structural changes in the retina.

The exact mechanism by which CQ and HCQ cause retinal toxicity is yet to be determined. Data from *in-vitro* and animal model studies suggest that the drugs accumulate in RPE cells, inhibit the recycling of all-trans-retinol and might result in photoreceptor degeneration.<sup>3,29</sup> However, recent data from SD-OCT scans in humans suggest an alternative sequence of events in which the primary site of toxicity is the photoreceptor layer, with secondary effect on RPE cells.<sup>30</sup>

Studies in humans point to the strong correlation between high daily doses of the drug, long duration of treatment and retinal toxicity.<sup>2,10</sup> In humans it is recommended that a daily CQ dose of 2.3 mg/kg should not be exceeded, and the risk for retinopathy rises dramatically after 10 years of treatment.<sup>2</sup> Penguins in the Tisch Family Zoological Gardens in Jerusalem regularly receive a weekly dose of 10 mg/kg during spring-summer months, and in the current study, the same dose of 10 mg/kg was given daily for 12 days prior to the first recording. The pharmacokinetics, pharmacodynamics and dose/toxicity relationship of CQ treatment were never evaluated in penguins, but our results suggest that this dose might be associated with an adverse effect of retinal toxicity. However, comparison of ERG data from birds in the treatment and off-treatment groups suggests that this effect might be at least partially reversible, and the animals' retinal function improves after treatment is stopped. Further studies are warranted to establish dose/response and dose/toxicity relationships that would enable effective anti-malarial CQ treatment with minimal adverse effects in penguins.

Baseline values for ophthalmic parameters, including intraocular pressure, Schirmer tear test, modified phenol red thread test, echobiometry, ocular surface bacterial flora and refractive error have previously been published for various penguin species.<sup>31–34</sup> However to our knowledge, this is the first report of full-field ERG recording in penguins. A recent study from North American zoos revealed that cataract is a prominent finding in aged penguins<sup>31</sup> and cataract surgery in penguins was shown to be successful and contributed to the welfare of captive penguins.<sup>16,35</sup> In order to minimize duration of anesthesia, no pre-operative ERG was recorded in



**FIGURE 3** Histopathology of the posterior segment of the eye of a 3.8-year-old female penguin that died of avian malaria. *Plasmodium* spp. infestation was found in the liver, lungs and brain, but there were no signs of inflammation, infection or degeneration in the eye. (A) A cross-section of the posterior aspect of the eye, showing the sclera (black arrow), retina (blue arrow), optic disk (black asterisk) and pecten (red arrows). H&E  $\times$  40. (B) A higher magnification of the retina (demarcated by the blue line), choroid (demarcated by the red line) and sclera (demarcated by the black line). H&E  $\times$  100. (C) A higher magnification of the retina. H&E  $\times$  400. GCL, ganglion cell layer; ILM, inner limiting membrane; INL, inner nuclear layer; IPL, inner plexiform layer; NFL, nerve fiber layer; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; PRs, photoreceptor outer segments; RPE, retinal pigment epithelium

the 21 birds operated by Church *et al.*<sup>16</sup> However, our study shows that such recordings are feasible and diagnostic in alert birds even without dark adaptation or pupil dilation.

There are several limitations to this study. First, recording of alert birds with undilated pupils enabled only a minimal protocol, without any dark or light adaptation. This surely had an influence on the responses recorded, including the lack of recognizable scotopic responses. This might also explain why the percent of measurable responses to high-intensity flashes is lower than to standard-intensity flashes, as alert birds became less cooperative toward the end of the recording. However, since conditions were identical in the recording of both the treatment and off-treatment groups, we believe the responses recorded are valid and comparable, and that the response attenuation in treated birds was indeed significant. Furthermore,

while recordings without pupil dilation or dark adaptation are not *lege artis*, we were able to prove the feasibility of diagnostic recordings of very short duration and handling time in non-dilated birds, an important safety consideration in ERG studies of avian wildlife,<sup>16,18,19</sup> which may prove useful for future researchers. Second, in order to minimize stress in the flock during the second recording (of the off-treatment group), we recorded the first six birds that were captured, rather than trying to catch each and every bird in the flock to confirm whether or not it has been recorded 18 months previously. Consequently, only three birds were recorded in both sessions, while three birds from the off-treatment group were not recorded previously. Therefore, it is possible that some of the differences observed between the groups are due to inter-bird differences and not the direct result of the treatment.



Third, pupils were not dilated for the recording, nor for an ophthalmoscopic examination. CQ and HCQ retinal toxicity in humans is associated with the appearance of a “Bull’s eye maculopathy” and other fundus abnormalities<sup>3</sup> and an ophthalmoscopic evaluation could have contributed to our understanding of the toxic effect of CQ on the retina. However, the histopathology of six eyes from three treated birds suggests that this examination may have been unremarkable. Fourth, the eyes we studied histopathologically were not recorded in the ERG study. Nonetheless, the fact that we studied six eyes of three birds from the same flock that were treated with the same CQ protocol in the years and weeks preceding their deaths, and the lack of retinal lesions in these eyes, supports the hypothesis that ERG deficits in treated birds are not correlated with long-term retinal pathology, and that these deficits are reversible. Another noteworthy limitation is the fact we did not record ERG in a true control group consisting of CQ naïve birds, as the zoo treats all birds annually. Therefore, we cannot establish baseline ERG values of CQ naïve birds and, consequently, cannot claim that responses of the birds in the off-treatment group have fully recovered with no cumulative damage. Finally, in the current study we used full-field ERG to assess the effect of the drug on retinal function. Data from human studies show that the mfERG can detect retinal dysfunction in CQ retinal toxicity even when the full-field ERG is normal,<sup>21,22</sup> and current recommendations are to use mfERG in screening for CQ retinal toxicity.<sup>10</sup> Nonetheless, the effect of treatment in the current study was substantial enough to cause a significant attenuation of full-field ERG responses.

In summary, the present study reveals a significant effect of treatment with CQ on captive African penguins’ retinal function and suggests that the effect might be reversible upon cessation of treatment. Because penguins in zoos and rehabilitation centers worldwide are regularly treated with CQ as prophylactic anti-malarial treatment, further studies are warranted to establish an effective dose with reduced adverse effects. The data presented here also suggest that ERG responses of captive penguins undergoing ERG for any indication (such as prior to cataract surgery), must be evaluated in light of their anti-malaria treatment status.

## CONFLICT OF INTEREST

None.

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