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SEROPREVALENCE OF MALARIAL ANTIBODIES IN GALAPAGOS PENGUINS (*SPHENISCUS MENDICULUS*)

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ABSTRACT: A parasite species of the genus *Plasmodium* has recently been documented in the endangered Galapagos penguin (*Spheniscus mendiculus*). Avian malaria causes high mortality in several species after initial exposure and there is great concern for the conservation of the endemic Galapagos penguin. Using a *Plasmodium* spp. circumsporozoite protein antigen, we standardized an enzyme-linked immunosorbent assay to test the level of exposure in this small population, as indicated by seroprevalence. Sera from adult and juvenile Galapagos penguins collected between 2004 and 2009 on the Galapagos archipelago were tested for the presence of anti-*Plasmodium* spp. antibodies. Penguins were also tested for the prevalence of avian malaria parasite DNA using a polymerase chain reaction (PCR) screening. Total seroprevalence of malarial antibodies in this sample group was 97.2%, which suggests high exposure to the parasite and low *Plasmodium*-induced mortality. However, total prevalence of *Plasmodium* parasite DNA by PCR screening was 9.2%, and this suggests that parasite prevalence may be under-detected through PCR screening. Multiple detection methods may be necessary to measure the real extent of *Plasmodium* exposure on the archipelago.

Introduced diseases such as avian malaria can severely impact the health of small populations and have been the cause of species extinctions (Warner, 1968; Atkinson et al., 1995, 2000). Island species, due to their geographic isolation, are thought to be especially susceptible to introduced diseases (Van Riper et al., 1986; Atkinson et al., 2000). The Galapagos penguin (*Spheniscus mendiculus*) is endemic to the Galapagos Islands, with a population of approximately 1,500 individuals (Jimenez-Uzcategui and Vargas, 2008), and is considered endangered (Birdlife International, 2010) because its population is perpetually small and experiences significant fluctuations in response to climate (Vargas et al., 2005). The Galapagos archipelago, located on the equator approximately 1,000 km west of continental Ecuador, preserves 95% of the species diversity known to have existed there (Gibbs et al., 1999). To date there have been no extinctions of endemic avian species on the archipelago; however, tourism and human population growth are creating threats to the long-term survival of native species, due in part to introduced pathogens including avian pox (*Avipoxvirus*) (Parker et al., 2011) and avian malaria (*Plasmodium*) (Wikelski et al., 2004; Parker et al., 2006; Levin et al., 2009).

Populations on geographically isolated islands are considered immunologically naïve, suggesting that their susceptibility to introduced pathogens should be high. For example, the relatively recent introduction of the mosquito (*Culex quinquefasciatus*) and avian malaria (*Plasmodium relictum*) to the Hawaiian Islands caused severe declines and extinctions of many avian species, particularly the native honeycreepers (*Drepanidinae*) in the mid-elevation range where *C. quinquefasciatus* are most concentrated (Warner, 1968; van Riper et al., 1986; Atkinson et al., 2000). In the Galapagos penguin, this vulnerability to infectious diseases is likely magnified due to its small population size and periodic bottlenecks (Vargas et al., 2005), low genetic diversity (Nims et al., 2008), and very low variation in the major histocompatibility complex (MHC) (Bollmer et al., 2007). Environmental stresses such as El Niño events may increase disease susceptibility of isolated populations during these periods, threatening the long-term survival of the species (Vargas et al., 2006).

Levin et al. (2009) determined by polymerase chain reaction (PCR) that a vector-borne blood parasite in the genus *Plasmodium* had a prevalence of 5% in Galapagos penguins (n = 362). There are 2 potential *Plasmodium* vectors on the archipelago, the native mosquito species *Aedes taeniorhynchus* and a non-native mosquito species, *Culex quinquefasciatus*, a known *Plasmodium* vector elsewhere that has become established on the Galapagos Islands since its introduction in the mid-1980s (Whiteman et al., 2005). The extent of the parasite's distribution and its potential long-term effect on the Galapagos penguin is especially important given the knowledge that these parasites (in particular *Plasmodium relictum* and *Plasmodium elongatum*) have resulted in high morbidity and mortality in other penguin species, both captive and wild (Stoskopf and Beier, 1979; Fix et al., 1988; Cranfield et al., 1990; Graczyk, Cranfield, McCutchan et al., 1994; Graczyk, Brossy et al., 1995; Graczyk, Cranfield et al., 1995). For example, malaria-related mortality (due to *P. relictum* and *P. elongatum*) of previously unexposed captive African black-footed penguins (*Spheniscus demersus)* has been recorded as high as 50 and 75% (Stoskopf and Beier, 1979; Cranfield et al., 1990). *Plasmodium* antibodies have been reported in captive adult African black-footed penguins that are seasonally exposed to an outdoor environment with competent *Culex* spp. mosquito vectors (*Culex pipiens* and *Culex restuans*) (Graczyk, Cranfield, Skjoldager et al., 1994). In captive juvenile African black-footed penguins (n = 24), within 5 wk after initial exposure to *P. relictum* and *P. elongatum* parasites seroprevalence of malarial antibodies was 100% with a 9% mortality rate (Graczyk, Cranfield, McCutchan et al., 1994). Females can pass these antibodies to their chicks, allowing for

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short-term protection from malarial infection (Graczyk, Cranfield, Shaw et al., 1994). Once maternal antibodies levels are no longer detectable, juveniles are then susceptible to infection; in captive African black-footed penguin chicks this occurred at 10 wk of age (Graczyk, Cranfield, Shaw et al., 1994).

In Hawaiian forest birds, acute infections cause rates of mortality up to 90% (Atkinson et al., 1995) and increase an individual's susceptibility to other stresses such as predators or environmental stress (Atkinson et al., 2000; Yorinks and Atkinson, 2000). After acute *P. relictum* infection, birds maintain low-level parasitemia for life and may relapse periodically (Cranfield et al., 1990; Atkinson and van Riper, 1991). These low-intensity chronic infections are not easily detected on blood smears and are detected irregularly through PCR screening (Atkinson, Dusek et al., 2001; Atkinson, Lease et al., 2001; Jarvi et al., 2002; Woodworth et al., 2005). Survival rates during reinfection are much higher because of acquired immunity to the parasite (Atkinson, Dusek et al., 2001; Atkinson, Lease et al., 2001; Jarvi et al., 2002; Woodworth et al., 2005). In the case of the extremely susceptible Hawaiian amakihi (*Hemignathus virens*), populations are recolonizing former habitats at low elevations despite the high prevalence of *P. relictum*, indicating an acquired immunity and possible host–parasite coevolution (Woodworth et al., 2005).

We present here the first study measuring seroprevalence of anti-*Plasmodium* spp. antibodies in the Galapagos penguins by enzyme-linked immunosorbent assay (ELISA). We also used PCR screening techniques to detect the prevalence of *Plasmodium* spp. in the same birds. While Levin et al. (2009) successfully detected the presence of *Plasmodium* spp. in the Galapagos penguin through PCR screening, determining anti-*Plasmodium* spp. antibody seroprevalence is necessary to give a more accurate measure of *Plasmodium* exposure in the population. *Plasmodium* gametocytes have yet to be detected in the penguins by blood smear examination (Levin et al., 2013). This absence of gametocytes, the final blood-borne stage that will become reproductive immediately after ingestion into the mosquito host (Valkiūnas, 2005), suggests that the Galapagos penguins may not be competent hosts because the parasite does not appear to complete its life cycle in this species. Another bird species on the archipelago is then acting as the reservoir for the parasite. Because of this, *Plasmodium* prevalence is also likely to be underdetected by PCR screening methods using peripheral blood as the source of DNA. Low parasite detection by PCR combined with low seroprevalence of malarial antibodies could indicate low exposure or that the population is highly susceptible to infection and most individuals are not surviving the initial *Plasmodium* infection. High seroprevalence in the population, however, would suggest that many or most penguins are surviving the infections and are therefore developing an effective level of acquired immunity (Atkinson and van Riper, 1991; Cranfield et al., 1994).

Although the penguin's range overlaps year-round with the ranges of potential mosquito vectors, it is likely that *Plasmodium* infection rates will fluctuate seasonally with higher transmission during the wet season when vectors are more abundant. Therefore, we expect juvenile seroprevalence to be lower than that of the adults considering they may not have been exposed prior to sampling. If the population had been exposed to *Plasmodium* for several years, when we compare seroprevalence from our core group of penguins sampled between 2008 and 2009 to a group of penguins sampled earlier (2004–2005), we predicted seroprevalence of anti-*Plasmodium* spp. antibodies would be similarly high in both penguin groups. Alternatively, if *Plasmodium* had only recently arrived before the 2004–2005 sampling, seroprevalence might be lower due to lack of exposure in the earlier sample group than in 2008–2009.

MATERIALS AND METHODS

Sample collection

Galapagos penguins were captured in hand nets along the coasts of Isabela and Fernandina islands in the Galapagos Islands (for field handling techniques and sample processing, see Travis et al., 2006). During 2 field seasons (March–September 2008 and July 2009) serum samples were collected at 8 sites (Fig. 1) from a total of 181 Galapagos penguins: 149 adults, 24 juveniles, and 8 individuals of undetermined age. Sites that were within 10 km of each other were combined for this study and listed as 1 site. Punta Espinosa and Cañones Sur are both sites that are a combination of 2 sites (see Fig. 1). Serum samples collected from 64 adult penguins during earlier field seasons (March and August 2004 and February 2005) are also used in this study (Travis et al., 2006). The 64 samples were a subset of the samples collected in 2004–2005 for which both serum and blood were available for testing.

Serologic analysis

Indirect ELISAs were performed using a KPL, Inc. BluePhos® system ELISA kit (Gaithersburg, Maryland) and Nunc brand Immulon II high-binding 96-well ELISA plates (Thermo Scientific, Rochester, New York). Plates were coated with 100 µl (2 µg/ml) of a circumsporozoite protein (CSP) antigen (GenScript Corp., Piscataway, New Jersey) and incubated at 4 C for 24 hr. The CSP is expressed on the surface of *Plasmodium* spp. sporozoites and asexual parasite stages, and this CSP antigen is recognized by antibodies against avian *Plasmodium* species (T. F. McCutchan, unpubl. data). Plates were then filled with bovine serum albumin (BSA) to block any remaining unbound sites. Next, 100 µl of sera from the sampled penguins diluted in BSA (1/100) were added in duplicate and incubated for 17 hr at 4 C.

A negative control and standard were added to every plate. The standard was also used as a positive control. The negative control, run in triplicate on each plate, was serum from chickens housed in a mosquito-free environment (gift from the Entomology section, Laboratory of Malaria and Vector Research, NIAID, National Institutes of Health, Bethesda, Maryland), diluted in BSA (1/100). The standard, run in duplicate serial dilution in BSA (1/100 to 1/1,600) on each plate, was a

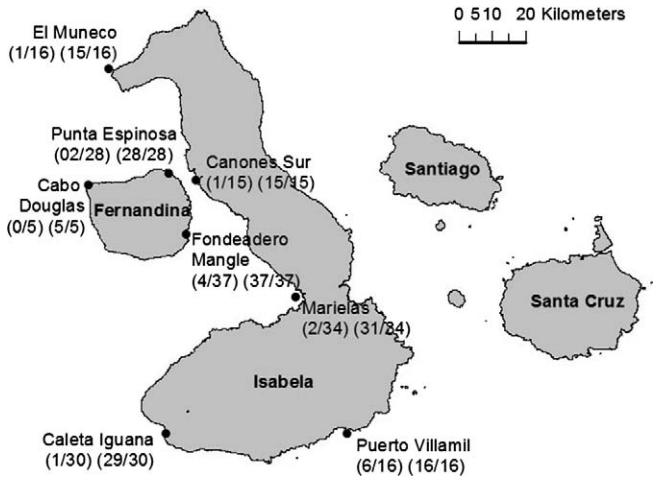


FIGURE 1. Map of 2008–2009 sample sites. Total of 8 sites located on Isabela and Fernandina islands. Numbers in parentheses show prevalence of parasite DNA by PCR and seroprevalence (no. PCR positive/total) (no. seropositive/total).

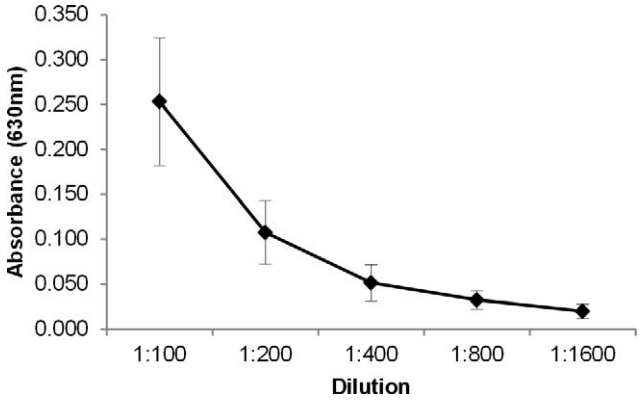


FIGURE 2. Standard. Pool of 3 penguins from the 2008 field season that amplify as *Plasmodium* on PCR, run in serial dilution from 1/100 to 1/1,600 on each plate. Curve includes the mean absorbance values across 10 plates ± SD.

pool of 3 adult PCR-positive Galapagos penguins collected in 2008 (Fig. 2).

Each well was then washed 3 times with 300 µl of imidazole-buffered saline with Tween®-20 wash solution diluted in water (1/20). Next, 100 µl of rabbit anti-*Spheniscus demersus* IgG (Spring Valley Laboratories, Inc., Woodbine, Maryland), conjugated to alkaline phosphatase diluted in BSA (1/1,000), was added to each well and incubated for 2 hr at room temperature. The plates went through a 2nd set of 3 washes with 300 µl of the wash solution. Next, 100 µl of BluePhos alkaline phosphatase substrate solution was added to each well. The plates were incubated for 95 min at room temperature and read (630 nm) on a VERSAmax microplate reader controlled by SOFTmax® Pro software version 4.7 (Molecular Devices Corp., Sunnyvale, California).

The standard was used to determine a positive cut-off absorbance value as well as to make adjustments for plate-to-plate variation. The positive cut-off was calculated as the mean + 3 standard deviations (SD) across 10 plates of the standard dilution at which the sample was visually indistinguishable from the negative controls (1/1,600 dilution). The mean absorbance value of the standard (1/100 dilution) of the 10 plates acted as the positive control and was used to make adjustments for plate-to-plate variation. Each individual plate mean standard (1/100 dilution) was compared to this value and absorbance values of all serum samples were adjusted by the difference.

Molecular screening

Using a standard phenol-chloroform extraction protocol, DNA was extracted from blood (Sambrook et al., 1989). A region of the parasite mitochondrial cytochrome *b* gene was amplified by PCR using 2 protocols: (1) primers published in Perkins and Schall (2002) following the protocol in Levin et al. (2009), and (2) primers published in Waldenström et al. (2004) following the modified protocol in Levin et al. (2011). Both positive and negative controls were always used. The positive control was a PCR-positive Galapagos penguin that amplified consistently and the negative control consisted of all the PCR reagents minus DNA.

Because the screening primers amplify both *Plasmodium* and *Haemoproteus* parasites, DNA sequencing was performed to identify the PCR-positives as *Plasmodium*. Using BigDye® terminator v3.1 cycle sequencing kits (Applied Biosystems, Carlsbad, California), PCR-positive individuals were sequenced in 10-µl reactions with a final primer concentration of 1 µM and followed a standard cycle sequencing program (35 cycles of 94 C for 30 sec, 50 C for 30 sec, 72 C for 30 sec, 1 cycle of 72 C for 10 min). Ethanol precipitation (1 µl each 3M NaOAc and 0.125M EDTA with 25 µl 100% ethanol per 20 µl PCR product) was used to clean sequencing reactions before sequencing on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, California). Sequences were analyzed individually using Seqman (Lasergene, DNASTAR, Inc., Madison, Wisconsin). DNA sequences were BLASTed against previously published *Plasmodium* spp. sequences from Galapagos penguins (GenBank JF833046, JF833047).

Data analysis

As the data were not normally distributed, nonparametric statistical analyses were performed using SPSS analytical software (Version 19, IBM, Armonk, New York). Mann–Whitney *U*-tests were used to evaluate differences in absorbance distributions by age and to compare the 2008–2009 sample set with the samples tested from 2004–2005. Because of small sample sizes at some sites and non-normality of the absorbance distributions among all 8 sites, a Kruskal-Wallis ANOVA was performed to compare absorbance distributions across sites. A paired *t*-test was performed to test for any difference in absorbance values of penguins that were captured in more than 1 field season. Statistical significance was set at $P < 0.05$.

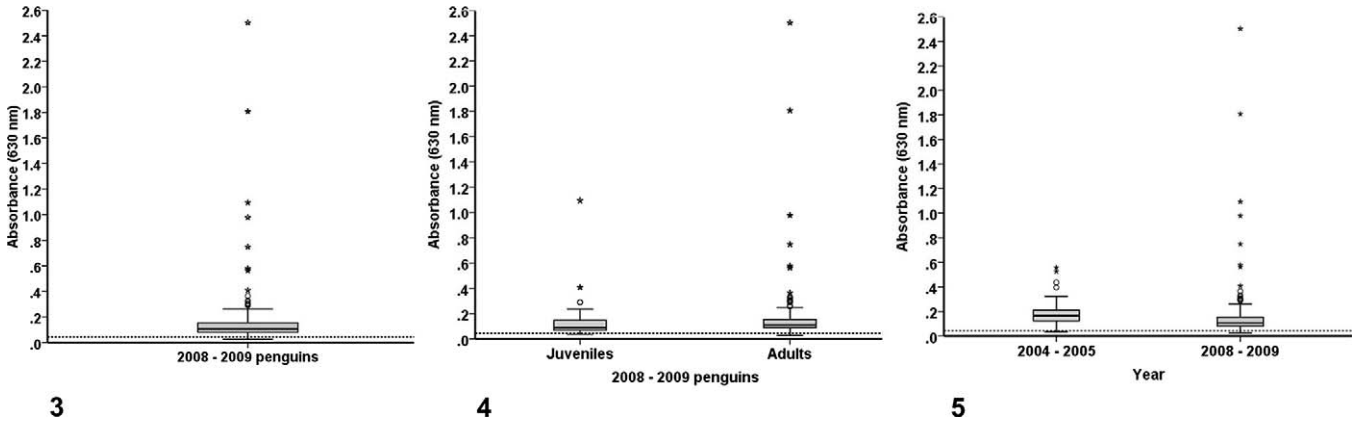
RESULTS

The range in absorbance values for the standard at 1/1,600 dilution was 0.010–0.037 with a mean ± 3 SD of 0.020 ± 0.024, establishing a positive cut-off absorbance value of 0.044. Any individual with an absorbance value at or above 0.044 was considered seropositive. The negative control absorbance values ranged from –0.009 to 0.009 with a mean absorbance value of 0.0 ± 0.003 SD.

For the core group of penguins sampled in 2008–2009, 176 individuals out of 181 were identified as seropositive (97.2% seroprevalence). Absorbance values ranged from 0.025 to 2.503 with a median absorbance value and interquartile range (IQR) of 0.108 (.074) (Fig. 3). Seroprevalence was similarly high across the penguins' range, ranging from 91.2 to 100% among sites (Table I). Kruskal-Wallis tests showed no significant difference among site absorbance value distributions ($H = 12.712$, $P = 0.079$). The data did, however, violate the following assumptions: (1) equal distribution shape between groups, and (2) small sample size variation among sites, increasing the probability of the test not rejecting the null hypothesis (Whitlock and Schluter, 2009).

Adult seroprevalence was 97.3% (n = 149) and absorbance values ranged from 0.025 to 2.503. The median absorbance value (with IQR) for adult penguins was 0.111 (0.067). Juvenile seroprevalence was 95.8% (n = 24); absorbance values for juveniles ranged from 0.035 to 1.093 with a median absorbance value (and IQR) of 0.088 (0.105). Seroprevalence for penguins of undetermined age (n = 8) was 100%; absorbance values for these individuals ranged from 0.044 to 0.173 with a median absorbance value and IQR of 0.098 (0.076). A Mann–Whitney *U*-test determined no significant difference in absorbance distributions between adult and juvenile penguins ($U = 1.54$, $P = 0.122$). Antibody distribution comparisons between adults and juveniles are shown in Figure 4. Descriptive statistics of adult and juvenile antibody distributions, as indicated by absorbance values, are shown in Table II.

Sixty-four adult penguins sampled in 2004 or 2005 were tested to determine seroprevalence prior to 2008–2009. Total anti-*Plasmodium* spp. antibody seroprevalence for penguins sampled in 2004 and 2005 was 96.8%, with only 2 individuals falling below the cut-off of an absorbance of 0.044. Absorbance values ranged from 0.032 to 0.558 with a median absorbance value (and IQR) of 0.166 (0.09). A Mann–Whitney *U*-test suggested a significant difference in absorbance distributions between penguins sampled in 2004–2005 and those sampled in 2008–2009 ($U = -4.471$, $P < 0.05$). The median absorbance value for 2004–2005 penguins was



FIGURES 3–5. **(3)** 2008–2009 absorbance distribution. Box plots displaying antibody levels, as shown by absorbance (630 nm), of 181 total penguins, 149 adults, 24 juveniles, and 8 penguins of undetermined age. Positive cut-off indicated by dotted line at an absorbance of 0.044. **(4)** 2008–2009 penguins by age group. Box plots displaying distribution of antibody levels, as shown by absorbance (630 nm), for juvenile and adult penguins. Juveniles (n = 24), adults (n = 149). Positive cut-off indicated by dotted line at an absorbance of 0.044. **(5)** Comparison over time. Distribution of antibody levels, as shown by absorbance (630 nm), of 64 penguins from the 2004–2005 field seasons as compared to 181 penguins from the 2008–2009 field seasons. Positive cut-off indicated by dotted line at an absorbance of 0.044.

higher (0.166) than for the 2008–2009 penguins (0.108). Antibody distributions for 2004–2005 and 2008–2009 penguins, based on absorbance values, are shown in Figure 5. Descriptive statistics for antibody distributions comparing these sample groups are shown in Table II.

Of the 181 penguins sampled in 2008–2009, 26 were recaptures, meaning they were previously sampled at some point between 2003 and 2005. Another 5 penguins that were first sampled in the 2008 field season were sampled again in 2009. Serum was available for only 10 of the recaptured penguins at both periods of sampling, and absorbance value comparisons for these penguins are shown in Table III. Results of a paired *t*-test run on log-transformed data determined no significant difference in mean absorbance values between years ($t = -0.859$, $P = 0.413$).

Molecular screening

Prevalence of *Plasmodium* spp. DNA, determined by PCR, was 9.4% (17/181). All but 1 PCR-positive penguin were adults. Results of DNA sequencing showed all 17 individuals sequenced as the same *Plasmodium* lineage previously described in Levin et al. (2009) (see GenBank JF833046, JF833047). Absorbance values for serum from these 17 penguins ranged from 0.067–1.807 with a median absorbance value and IQR of 0.129 (0.173). Absorbance values of the PCR-negative penguins (n = 164) ranged from 0.025–2.503 with a median absorbance value and IQR of 0.107 (0.068). A Mann–Whitney *U*-test determined no significant difference in absorbance distributions between PCR-positive and PCR-negative penguins ($U = 1.449$, $P = 0.147$). Only 2/64 penguins (3.1%) sampled from 2004 and 2005 were PCR-positive. The absorbance values for the 2 PCR-positive individuals were 0.396 and 0.558. DNA sequencing determined that both individuals were infected with the same *Plasmodium* lineage previously described in Levin et al. (2009) (see GenBank JF833046, JF833047).

TABLE I. Seroprevalence of anti-*Plasmodium* spp. antibodies in 181 Galapagos penguins. Positive for anti-*Plasmodium* spp. antibodies is determined as an absorbance value equal to or above 0.044 (630 nm) by ELISA. This value is equal to the mean absorbance + 3 SD of the standard at the 1:1,600 dilution.

	2008–2009			
Site	Total no.	Positive no.	Positive (%)	Absorbance range
El Muneco	16	15	93.8	0.025–0.749
Punta Espinosa	28	28	100	0.049–0.295
Cabo Douglas	5	5	100	0.055–0.977
Cañones Sur	15	15	100	0.047–0.212
Fondeadero Mangle	37	37	100	0.060–0.578
Islas Marielas	34	31	91.2	0.035–2.503
Caleta Iguana	30	29	96.7	0.043–0.249
Puerto Villamil	16	16	100	0.049–1.807
Total	181	176	97.2	0.025–2.503

DISCUSSION

High seroprevalence (97.2%) of anti-*Plasmodium* spp. antibodies occurs in the Galapagos penguins sampled in 2008–2009, despite low detection of *Plasmodium* spp. DNA by PCR screening (9.4%). Seroprevalence ranged from 91.2 to 100% among sample sites, which is expected given that the Galapagos penguins move regularly throughout their range (Nims et al., 2008). Similarly, high seroprevalence (96.8%) was determined for the penguins sampled in 2004 and 2005 whereas detection of parasite DNA by PCR was also very low (3.1%). PCR amplifies haemosporidian parasite DNA in the circulating blood, regardless of the parasite's life stage, and therefore may not be detecting gametocytes (Valkiūnas, 2011). Low rates of parasite detection in blood by PCR, along with the lack of gametocyte detection in blood smears, indicates that the parasite may not be completing its life cycle in the penguins. PCR results for 2004–2005 samples in this study show low parasite prevalence similar to that reported in Levin et al. (2009) (5% prevalence, n = 362). In our study, penguins sampled in 2004–2005 were tested when serum was available for ELISA testing. The penguins are likely infected, but

TABLE II. Descriptive statistics for antibody distributions, as shown by absorbance, of all individuals tested, separated by year and age class.

Year	Statistics	n	Mean	Median	SD	IQR
2008–2009	Total	181	0.167	0.108	0.252	0.074
	Adult	149	0.171	0.111	0.264	0.067
	Juvenile	24	0.162	0.088	0.217	0.105
	Unknown age	8	0.105	0.098	0.046	0.076
2004–2005	Total (all Adult)	64	0.184	0.166	0.101	0.090

the absence of gametocytes suggests the possibility of abortive parasite development during the exoerythrocytic stage of development (Olias et al., 2011; Valkiūnas, 2011). The bird species acting as the reservoir for the parasite has yet to be determined on the archipelago, and this is the subject of current investigation.

Given that positivity for malarial antibodies requires just a single contact with the parasite, and that an acquired immunity develops after the first exposure to the parasite (Atkinson and van Riper, 1991; Cranfield et al., 1994; Graczyk, Cranfield, Skjoldager et al., 1994), our results suggest that parasite intensities in most individuals are too low to be amplified by PCR but that the penguins have been broadly exposed to the parasite. High antibody levels in some individuals suggest possible current infections. Jarvi et al. (2002) reported serological methods used to determine antibody seroprevalence (97%) to be more sensitive in detecting low-intensity chronic *P. relictum* infections in Hawaiian passerines (*Hemignathus virens*) than either microscopy (27%) or PCR-based methods (61–84%).

Seroprevalence between juveniles and adults was predicted to differ, which would have suggested that the juveniles are more susceptible to mortality from infection, but our results suggest no difference in antibody distributions based on absorbance values. Any juveniles not yet infected, following a period of protection by maternally transmitted antibodies, would have tested seronegative for malarial antibodies. The presence of maternally transmitted antibodies is ruled out as an explanation for high seroprevalence in juveniles in this study because each juvenile sampled was already fledged and, therefore, beyond the lifespan of maternally transmitted antibodies in other penguins (Graczyk, Cranfield, Shaw et al., 1994). Malarial antibodies have not yet been tested in Galapagos penguin chicks. Assuming that the parasite is established in the population, though, recently hatched Galapagos penguin chicks should have detectable malarial antibody levels. In New Zealand, yellow-eyed penguin chicks (*Megadyptes antipodes*) remained unaffected after approximately 150 adult penguins died of avian malaria over a 2-mo period (Graczyk, Cockrem et al., 1995). Chick survival was likely due to high maternally transmitted antibody titers that protected them from the outbreak (Graczyk, Cockrem et al., 1995).

The majority of documented cases of high mortality and morbidity due to malaria outbreaks in penguins occur in captive environments where immunologically naïve penguins are introduced to outdoor environments with *Culex* sp. mosquitoes that transmit *P. relictum* and *P. elongatum*. Typically, these penguins are wild-caught individuals relocated from climates that lack *Plasmodium* sp. parasites or were only seasonally exposed to the parasite during migration (Stoskopf and Beier, 1979; Fix et al., 1988; Cranfield et al., 1990; Graczyk, Brossy et al., 1995). The penguins that do not survive *Plasmodium* spp. infections die quickly after initial exposure (Graczyk, Cranfield, Skjoldager et al., 1994). Clinical disease and cause of mortality due to *Plasmodium* infection is, in these cases, easier to observe and diagnose (Graczyk, Cranfield, McCutchan et al., 1994), yet even in captive populations, signs of parasitemia and clinical disease are often absent (Fix et al., 1988).

All of the Galapagos penguins sampled in our study were in apparently good health and did not show signs of clinical disease. If they are dying from infection, the carcasses are unlikely to be found because they would either be quickly eaten by predators or scavengers or washed away, increasing the challenge of determining if any are dying due to disease (Wobeser and Wobeser, 1992; Bennett et al., 1993). Our results suggest that individuals are

TABLE III. Absorbance value comparisons of individuals sampled in more than 1 yr. Positive for anti-*Plasmodium* antibodies is determined as an absorbance value equal to or above 0.044 (630 nm) by ELISA. This value is equal to the mean absorbance + 3 SD of the standard at the 1:1,600 dilution.

	Recapture comparisons		Absorbance values		
Site	Penguin ID	2004–2005	2008	2009	Difference (%)
Cabo Douglas	2506	0.167	—	0.103	−6.4
Caleta Iguana	1671	0.146	0.043	—	−10.3
Fondeadero Mangle	2505	0.165	0.093	—	−7.2
Fondeadero Mangle	5471	—	0.143	0.125	−1.8
Fondeadero Mangle	5480	—	0.092	0.235	14.3
Fondeadero Mangle	1081	—	0.101	0.082	−1.9
Islas Marielas	1630	0.325	0.113	—	−21.2
Islas Marielas	2519	0.077	0.239	—	16.2
Puerto Villamil	5516	—	0.096*	0.100*	0.4
Punta Espinosa	5488	—	0.134	0.102	−3.2

* Indicates individuals that tested as positive by PCR.

able to survive infections, given that many penguins were recaptured in seemingly good health on 2, and in 1 case 3, different occasions across several years. High seroprevalence in samples collected between 2004 and 2005 (96.8%) indicates that penguins were first exposed to the parasite earlier than 2004. The median antibody levels were significantly higher in 2004–2005 than in 2008–2009. If parasite infections during 2004–2005 were very recent, higher antibody production may be due to the initial inflammatory stage in the penguins after initial exposure. However, fluctuations in the distribution of antibody levels within the population over time is expected given the number of environmental stressors, such as fluctuations in weather conditions or changes in prey abundance, that might affect individual susceptibility. We suspect that these temporal differences are attributable to normal antibody fluctuations.

The Galapagos penguins continue to be heavily impacted by warm El Niño events that cause surface water temperatures to increase, food supplies to decrease (Vargas et al., 2006), and rainfall to increase on land (Snell and Rea, 1999). Following the last 2 strong El Niño events, the penguin population fell by 77% (1982–1983) and 65% (1997–1998) (Vargas et al., 2006). If stress is shown to increase susceptibility to *Plasmodium* infection (Graczyk, Brossy et al., 1995), the stress of an El Niño event that may trigger a recurrence of *Plasmodium* infection could reduce survivorship in this species. Compared to other penguin species, the Galapagos penguin has lower genetic diversity (Nims et al., 2008) as well as very low MHC variability (Bollmer et al., 2007). We now know that pathogens, including *Chlamydophila psittaci*, *Toxoplasma gondii*, and microfilariae, have also been reported in the penguins (Travis et al., 2006; Merkel et al., 2007; Deem et al., 2010), and the combination of environmental pressure and co-infections of parasitic agents may increase the penguins' risk of mortality and susceptibility to *Plasmodium* infections. However, over the time period of our sampling the penguins show high seroprevalence, suggesting they are surviving with these infections.

Variations in *Plasmodium* exposure rates have been reported in other species of penguins based on whether their geographic range overlaps that of competent vectors, with higher seroprevalence of *Plasmodium* antibodies in more-northern ranging penguin species (Graczyk, Cockrem et al., 1995; Graczyk, Cranfield et al., 1995). The suspected *Plasmodium* spp. vector on the Galapagos archipelago is the well-established mosquito species *C. quinquefasciatus* (Whiteman et al., 2005). The range of this freshwater-breeding mosquito on the archipelago is limited, as there are very few fresh-water sources outside of human-inhabited areas. *Aedes taeniorhynchus*, the only recorded native species of mosquito on the islands that is known to bite birds (Bataille et al., 2009), may also be a potential vector of *Plasmodium* spp. Given that *A. taeniorhynchus* breeds in brackish water and that some of the penguins testing positive for *Plasmodium* spp. infection were sampled in areas lacking regular fresh-water sources, this native species cannot be discounted as a possible vector.

Extremely high seroprevalence of *Plasmodium* spp. antibodies strongly suggest that this species of penguin is surviving in the face of malarial infections regardless of whether they are competent hosts for the parasite. With the amount of data now available for this population, we recommend that a meta-analysis of the penguins' disease status be conducted and compared with mark–recapture data to see what individual- and population-level impacts these pathogens alone, or as co-infections, might be having. Understanding the transmission dynamics (reservoir species and vector species) of this parasite on the archipelago is essential to the long-term conservation of the Galapagos penguin and other susceptible endemic avian species.

Because the penguins are seemingly healthy, and given such broad exposure to the *Plasmodium* parasite, the parasite is not likely very recently introduced. A comparison of population antibody levels over time will give a clearer picture of disease-related fluctuations in the population.

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