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Seroprevalence of Malarial Antibodies in Galapagos Penguins (*Spheniscus mendiculus*)

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Seroprevalence of Malarial Antibodies in Galapagos Penguins (*Spheniscus mendiculus*)

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Advisory Committee

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

ABSTRACT: Introduced diseases such as avian malaria can severely impact the health of small populations and have been the cause of species extinctions. Island species are thought to be more highly susceptible to introduced diseases. These populations have likely evolved in the absence of various pathogens making them immunologically naïve to diseases that occur regularly in mainland species. The Galapagos archipelago still preserves 95% of its species diversity known to have occurred there with most of its species being endemic to the islands. The introduction of Haemosporidian parasites in the genus *Plasmodium*, which cause avian malaria, have had detrimental impacts on naïve populations in other island systems. Until recently, avian malarial parasites had not been detected in the Galapagos avifauna; however, the presence of *Plasmodium* parasites has been documented in the endangered Galapagos penguin (*Spheniscus mendiculus*). Because avian malaria (*Plasmodium relictum*) causes high mortality in other avian species after initial exposure, there is concern for the conservation of the endemic Galapagos penguin. Using a *Plasmodium* spp. circumsporozoite protein antigen, we have standardized an enzyme-linked immunosorbent assay (ELISA) to test the level of exposure to the parasite in this species, as indicated by seroprevalence. Low seroprevalence would be consistent with high mortality, whereas high seroprevalence may indicate low *Plasmodium*-induced mortality under normal conditions. Serum from Galapagos penguins collected between 2004 and 2009 on the Galapagos archipelago was tested for the presence of anti - *Plasmodium* spp. antibodies. Penguins were also tested for prevalence of avian malaria parasites, determined by polymerase chain reaction

(PCR). Total seroprevalence of malarial antibodies in this sample group was 97.2%, while total prevalence of *Plasmodium* parasites by PCR screening was 9.2%. This large discrepancy suggests high exposure to the parasite and low *Plasmodium* - induced mortality, at least under normal environmental conditions. The results of this study also suggest that parasite prevalence may be under-detected through PCR screening and multiple detection methods are necessary to better understand the extent of *Plasmodium* on the archipelago. It is extremely important to understand the distribution of this parasite on the islands and control any invasive threats before irreversible damage is done.

Key words: seroprevalence, *Plasmodium*, *Spheniscus mendiculus*, avian malaria, ELISA, antibody, Galapagos penguin

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INTRODUCTION

The Galapagos penguin (*Spheniscus mendiculus*) is endemic to the Galapagos Islands with a population of approximately 1500 individuals (Jimenez – Uzcatogui and Vargas, 2008) and is considered endangered (IUCN 2010; Birdlife International, 2011) 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 1000 km west of continental Ecuador, still 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*) and avian malaria (*Plasmodium*) (Wikelski et al., 2004; Parker et al., 2006, 2011; Levin et al., 2009).

Island populations are considered immunologically naïve, suggesting that their susceptibility to introduced pathogens should be high (e.g., Hawaiian avifauna: Warner, 1968; van Riper et al., 1986; Atkinson et al., 2000). For example, the relatively recent introduction of mosquitos (*Culex quinquefasciatus*) and avian malaria (*Plasmodium relictum*) to the Hawaiian Islands caused severe declines and extinctions of many avian species, particularly in the native honeycreepers (*Drepanidinae*), from the mid-elevation

range where *C. quinquefasciatus* are most concentrated (van Riper et al., 1986; Atkinson et al., 2000). The threat that new pathogens pose to isolated, endemic island species is of great concern. In the Galapagos penguin, this inherent susceptibility to infectious diseases is likely magnified due to the species' small population size and periodic bottlenecks, 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 Nino Southern Oscillation events (ENSO), may increase their disease susceptibility during these periods, threatening the long term survival of the species.

Levin et al. (2009) determined by polymerase chain reaction (PCR) that the vector-borne blood parasite in the genus *Plasmodium* had a prevalence of 5% in Galapagos penguins (n = 362). There are two potential *Plasmodium* vectors on the archipelago. One of which is the non-native mosquito species, *Culex quinquefasciatus*, a known *Plasmodium* vector elsewhere which has become established on the Galapagos Islands after first being introduced in the mid – 1980s (Whiteman et al., 2005). Gaining a complete understanding of the extent of the *Plasmodium* parasite's distribution and its potential long-term effect on the Galapagos penguin is especially important given the knowledge that this parasite (specifically, *P. relictum* and *P. elongatum*) has 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 et al., 1994c; Graczyk et al., 1995a; Graczyk et al., 1995b). For example, malaria-related mortality (due to *P. relictum* and *P. elongatum*) of previously unexposed captive African black-footed penguins (*Spheniscus demersus*) has been reported to be as high as 50 and 75% (Stoskopf and Beier, 1979; Cranfield et al., 1990). *Plasmodium* antibodies have been reported in

adult African black-footed penguins that are seasonally exposed to an outdoor environment with a competent mosquito vector (Graczyk et al., 1994a). In captive, juvenile African black-footed penguins (n=24), within five weeks after initial exposure to *P. relictum* and *P. elongatum* parasites, seroprevalence of malarial antibodies was 100%, with a 9% mortality rate (Graczyk et al., 1994c). Females can pass these antibodies to their chicks, allowing for short-term protection from malarial infection (Graczyk et al., 1994b). 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 weeks of age (Graczyk et al., 1994b).

After acute *Plasmodium relictum* infection, birds maintain low-level parasitemia for life and may relapse periodically (Cranfield et al., 1990; Atkinson and van Riper, 1991). In Hawaiian forest birds, acute infections cause high rates of mortality (up to 90%) (Atkinson et al., 1995) and increase an individual's susceptibility to other stresses (e.g. predators or environmental stress) (Atkinson et al., 2000; Yorinks and Atkinson, 2000). However, individuals that survive acute infections acquire low-intensity chronic infections that are then not detected on blood smears and irregularly detected through PCR screening (Atkinson et al., 2001a; Atkinson et al., 2001b; Jarvi et al., 2002; Woodworth et al., 2005). Survival rates during reinfection are much higher because of acquired immunity to the parasite (Atkinson et al., 2001a; Atkinson et al., 2001b; Jarvi et al., 2002; Woodworth et al., 2005). And 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 a developed 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 use PCR screening techniques to detect the prevalence of *Plasmodium* parasites in the same birds. While Levin et al. (2009) successfully detected the presence of *Plasmodium* in the Galapagos penguin through PCR screening, determining anti - *Plasmodium* spp. antibody seroprevalence is necessary given that the parasite has yet to be detected by blood smear examination and it appears that PCR is under-detecting parasite prevalence in the population because the parasite may not be completing its life cycle in this host species (P. G. Parker, unpubl. data). Low parasite detection by PCR and low seroprevalence of malarial antibodies would indicate that the population is highly susceptible to infection and most individuals are not surviving the initial *Plasmodium* infection. High seroprevalence in the population, however, suggests that many or most penguins are surviving the infections, at least under normal environmental conditions (e.g., not El Nino years) and therefore developing a level of acquired immunity (Atkinson and van Riper, 1991; Cranfield et al. 1994). The use of multiple parasite detection methods will increase our understanding of true *Plasmodium* prevalence in the population.

We predict high seroprevalence of anti – *Plasmodium* spp. antibodies in adult penguins, indicating that the population has been broadly exposed. The potential for parasite transmission by an adult penguin at some point in a given year is high since the penguin’s range overlaps year - round with the ranges of potential mosquito vectors and because the penguins move regularly throughout their range (Nims et al., 2008) increasing their chance of becoming infected. Adults have most likely been exposed for

multiple seasons. We predict that *Plasmodium* infection rates fluctuate seasonally with higher transmission during the wet season, when vector populations are more abundant. Therefore, it is possible juvenile penguins were not exposed prior to sampling and seroprevalence will be low among this group. These young penguins are also no longer protected from infection by maternal antibodies, increasing their susceptibility to infection. Maternally transmitted antibodies only protect chicks for a short period after hatching (Graczyk et al., 1994b). Juveniles, if any, that test positive for anti - *Plasmodium* spp. antibodies are expected to have high antibody levels, indicating current infection because of recent exposure. If the population has, in fact, been exposed to *Plasmodium* for several seasons, 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 expect seroprevalence of anti - *Plasmodium* spp. antibodies to be similarly high in both penguin groups. Alternatively, if it had only recently arrived before the 2004-2005 sampling, seroprevalence may be lower in that sample group than in 2008-2009.

MATERIALS & METHODS

Sample collection

Galapagos penguins were captured in hand nets along the coasts of Isabela and Fernandina islands in the Galapagos Islands (see Travis et al., 2006 for field handling techniques and sample processing). During two field seasons (March – September 2008 and July 2009), serum samples were collected at 8 sites (Figure 1) from a total of 181 Galapagos penguins: 149 adults, 24 juveniles and 8 individuals of undetermined age.

Sites that were within 10 kilometers of each other were combined for this study and listed as one site. Punta Espinosa and Cañones Sur are both sites that are a combination of two sites (see Figure 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.

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 hours. The CSP is expressed on the surface of *Plasmodium* 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 hours 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 1/1600) on each plate, was a pool of 3 adult PCR positive Galapagos penguin collected in 2008 (Figure 2).

Each well was then washed 3 times with 300 μ l of Imidazole buffered saline with Tween-20 wash solution diluted in water (1/20). Next, 100 μ l of rabbit anti-*Spheniscus demersus* IgG (Spring Valley Laboratories, Inc., Woodbine, Maryland) conjugated to alkaline phosphatase, diluted in BSA (1/1000), was added to each well and incubated for two hours at room temperature. The plates went through a second set of three washes with 300 μ l of the wash solution. Next, 100 μ l of BluePhos® alkaline phosphatase substrate solution was added to each well. The plates were incubated for 95 minutes at room temperature and read (630nm) 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) of the standard dilution across 10 plates at which the sample was visually indistinguishable from the negative controls (1/1600 dilution). The mean absorbance value of the standard (1/100 dilution) of the 10 plates acted as the positive control and was used for plate-to-plate variation adjustments. 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 two 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.

DNA sequencing was performed to identify the PCR - positives as *Plasmodium* because the screening primers amplify both *Plasmodium* and *Haemoproteus* parasites. Using BigDye terminator v3.1 cycle sequencing kits (Applied Biosystems), PCR - positive individuals were sequenced in 10 μ L reactions with a final primer concentration of 1 μ M and following a standard cycle sequencing program (35 cycles of 94° 30", 50° 30", 72° 30", 1 cycle of 72° 10'). Ethanol precipitation (1 μ L each 3M NaOAc and 0.125M EDTA with 25 μ L 100% ethanol per 20 μ L PCR product) was used to clean sequencing reactions before sequencing on an ABI 3100 automated sequencer. Sequences were analyzed individually using Seqman (Lasergene). DNA sequences were BLASTed against previously published *Plasmodium* sequences from Galapagos penguins (GenBank accession numbers: JF833046, JF833047).

Data analysis

As the data were not normally distributed, nonparametric statistical analyses were performed, using SPSS analytical software (Version 19, IBM, Armonk, NY). Mann-Whitney U tests were used to evaluate differences in absorbance distributions by age as well as to compare our sample set with the samples tested from 2004-05. Because of small sample sizes at some sites and non-normality of the absorbance distributions among all eight 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 one field season.

Statistical significance was set at $P < 0.05$.

RESULTS

The range in absorbance values for the standard at 1/1600 dilution was 0.010 – 0.037 with a mean \pm 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. Five out of 181 total penguins fell below the positive cut-off of 0.044. 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, a total of 176 individuals of 181 were determined seropositive, yielding anti - *Plasmodium* antibody seroprevalence of 97.2%. Absorbance values ranged from 0.025 to 2.503 with a median absorbance value and interquartile range (IQR) of 0.108 (.074) (Figure 3). Three individuals had very high absorbance values compared to other individuals tested suggesting possible current infections. The mean absorbance value among these three individuals was 1.801 ± 0.705 SD with a median absorbance value of 1.807. All three penguins were adults and were collected at two sites: two at Marielas off the coast of Isabela Island and one at Puerto Villamil, on southern Isabela Island (see Figure 1). The penguin sampled at Puerto Villamil was the only individual, of the three, that tested positive for parasite DNA by PCR.

Seroprevalence was similarly high across the penguins' range, ranging from 91.2% to 100% among sites (Table 1). Kruskal-Wallis tests showed no significant difference among site absorbance value distributions ($H = 12.712$, $P = 0.079$) (see Figure 4). The data did, however, violate the following assumptions: 1) equal distribution shape between groups and 2) large 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 and IQR for adult penguins was 0.111 (0.067). Juvenile seroprevalence was 95.8% ($n = 24$) with only one seronegative individual; 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 ($U = 1.54$, $P = 0.122$). Frequency distribution comparisons between adults and juveniles are shown in Figure 5. Descriptive statistics of adult and juvenile antibody distributions, as shown by absorbance, are shown in Table 2.

Sixty-four adult penguins sampled in 2004 or 2005 were tested to determine seroprevalence prior to 2008 - 09. Total anti - *Plasmodium* spp. antibody seroprevalence for penguins sampled in 2004 and 2005 was 96.8%, with only two 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 suggests a significant difference in absorbance distributions between penguins sampled in

2004 - 05 and those sampled in 2008-09 ($U = -4.471$, $P < 0.05$). The median absorbance value for 2004 - 05 penguins was higher (0.166) than for the 2008 - 09 penguins (0.108). Antibody distributions for 2004 – 05 and 2008 – 09 penguins, based on absorbance values, are shown in Figure 6. Descriptive statistics for antibody distributions, as shown by absorbance, comparing these sample groups are shown in Table 2.

A total of 26/181 penguins sampled in 2008-2009 are recaptures, meaning they were previously sampled at some point between 2003 and 2005. Another five penguins that were first sampled in the 2008 field season were sampled again in 2009. Serum was only available for 10 of the recaptured penguins at both periods of sampling and absorbance value comparisons for these penguins are shown in Table 3. All penguins in Table 3 were recaptured at the same site of original sampling. Antibody levels, as shown by absorbance, of seven penguins decreased over time. Mean decrease in absorbance for these seven individuals was 0.074 ± 0.068 SD. The mean increase in absorbance values for the three individuals whose antibody levels increased over time is 0.103 ± 0.086 SD. Results of a paired t-test run on log transformed data determined no significant difference in mean absorbance values between years ($t = -0.859$, $P = 0.413$). One individual (ID 1081), first sampled in 2003, then resampled in 2008 and again in 2009 tested negative for *Plasmodium* DNA by PCR in each year sampled. Serum was not available from 2003 to test this individual for anti – *Plasmodium* spp. antibodies; however, serum from this penguin in both the 2008 and the 2009 field seasons was seropositive for anti – *Plasmodium* spp. antibodies (see Table 3), with a 1.9% decrease in absorbance from 2008 to 2009. Another penguin (ID 5516), first sampled in 2008 and then again in 2009, tested positive for the presence of *Plasmodium* DNA by PCR as well as seropositive in both

years. Anti – *Plasmodium* spp. antibodies, as indicated by absorbance, in this individual increased by 0.4% (0.096 to 0.100) from 2008 to 2009.

Molecular Screening

Prevalence of *Plasmodium* DNA, determined by PCR, was 9.4% (17/181); 6 penguins tested positive only with Waldenström et al. (2004) primers, 3 only with Perkins and Schall (2002) primers, and 8 penguins tested PCR positive using both screening methods (Table 4). All but one PCR - positive penguin were adults. Results of DNA sequencing show all 17 individuals sequenced as the same *Plasmodium* lineage previously described in Levin et al. (2009) (see GenBank accession numbers: 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). Interestingly, the individual with the highest absorbance value (2.503) did not test positive for parasite DNA by PCR screening. The remaining PCR - negative penguin (n = 164) absorbance values ranged from 0.025 – 1.093, with a median absorbance value and IQR of 0.107 (0.068). Only 2/64 penguins (3.1%) sampled from 2004 and 2005 were PCR - positive using both Waldenstrom et al. (2004) and Perkins and Schall (2002) primers. The absorbance values for the two PCR - positive individuals were 0.396 and 0.558. Results of DNA sequencing determined both individuals sequenced as the same *Plasmodium* lineage previously described in Levin et al. (2009) (see GenBank accession numbers: JF833046, JF833047).

DISCUSSION

Here we determined high seroprevalence (97.2%) of anti - *Plasmodium* spp. antibodies in the Galapagos penguins sampled in 2008 - 09, despite low detection of *Plasmodium* parasite 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 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). This along with the lack of gametocyte detection in blood smears indicates that the parasite may not be completing its life cycle in the penguins. The penguins are likely infected but the absence of gametocytes suggests the possibility of abortive parasite development during the exoerythrocytic stage of development (Olias et al., 2011; Valkiūnas, 2011).

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 et al., 1994a), our results suggest that parasite intensities in most individuals are too low to be amplified by PCR but the penguins have been broadly exposed to the parasite and are most likely not competent hosts. Jarvi et al. (2002) reported serological methods used to determine antibody seroprevalence (97%) to be more sensitive in detecting low-intensity chronic *Plasmodium 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 infection due to lack of previous exposure, 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 fallen below the positive cut-off absorbance value of 0.044. Our results suggest that juveniles are likely surviving initial infection and may become infected quickly after the maternally transmitted antibodies are no longer providing protection. 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. Maternally transmitted anti - *Plasmodium* spp. antibodies have not yet been tested for in Galapagos penguin chicks. Assuming that the parasite is established in the population, though, recently hatched Galapagos penguin chicks should have high anti - *Plasmodium* spp. maternal antibody levels. These maternally transmitted antibodies are present in African black - footed chicks in very high levels for approximately 10 weeks after hatching (Graczyk et al., 1994b). In New Zealand, Yellow-eyed penguin chicks (*Megadyptes antipodes*) remained unaffected after approximately 150 adult penguins died over a two month period, the cause determined to be avian malaria (Graczyk et al., 1995c). Chick survival was likely due to high maternally transmitted antibody titers that protected them from the outbreak (Graczyk et al., 1995c).

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. mosquitos 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 et al., 1995a). The penguins that do not survive *Plasmodium* infections die quickly after initial exposure, before they develop the appropriate humoral response to infection (Graczyk et al., 1994a). Clinical disease and cause of mortality due to *Plasmodium* infection is, in these cases, easier to observe and diagnose after initial infection (Graczyk et al., 1994c), yet even in captive populations, signs of parasitemia and clinical disease are often absent (Fix et al., 1988). Only 1/38 wild-caught Magellanic penguins (*S. magellanicus*) that died of malaria (*P. relictum*) at an Iowa zoo had detectable parasitized erythrocytes by blood smear examination (13% infected cells; 11% meronts, 2% gamonts) (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 are either quickly eaten by predators or scavengers, or are washed away, increasing the challenge of determining if any are becoming sick due to disease (Bennett et al., 1993). Our results suggest that individuals are able to survive infection given that many penguins were recaptured on two and, in a single case, three different occasions across several years in seemingly good health. High seroprevalence in samples collected between 2004 and 2005 (96.8%) indicates that penguins were first exposed to the parasite earlier than 2004, and these penguins were also reported to have been in seemingly good health (Travis et al., 2006). The median antibody levels between the two sets of years were determined to be significantly higher

in 2004 – 05 than 2008 – 09 (Mann-Whitney U, $U = -4.471$, $P < 0.05$). 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 stress levels and susceptibility.

High seroprevalence suggest low *Plasmodium* – related mortality in the population under normal environmental conditions, but what effect is *Plasmodium* having during stressful conditions? The Galapagos penguins are heavily impacted by El Niño Southern Oscillation (ENSO) events that cause surface water temperatures to increase and food supplies to decrease (Vargas et al. 2006). Following the last two strong ENSO events, the penguin population crashed by 77% (1982-83) and 65% (1997-98) (Vargas et al., 2006). Given that the ENSO events drastically decrease food supply for the penguins, and because stress is shown to increase susceptibility to *Plasmodium* infection (Graczyk et al., 1995a), the stress of an ENSO event that may trigger a recurrence of *Plasmodium* infection could be devastating for this already endangered species. Previous collapses during ENSO events have been attributed to starvation (Vargas et al., 2006), but perhaps some of the mortality may have been due to a combination of a recurrence of *Plasmodium* infection and the increase of environmental stress on the penguins. If the 1982-83 ENSO event coincided with the establishment of the mosquito vector *Culex quinquefasciatus* on the islands (first documented in the 1980's: Peck et al., 1998), high environmental stress may have increased the level of malarial infections in the penguins, and the combined effects of ENSO and *Plasmodium* introduction could have caused the extreme population decline during that event. Those that survived the initial infection would have been the older, healthier individuals that had then developed some immunity.

And as ENSO events increase in frequency, the frequency of *Plasmodium* – related relapse and mortality could also increase.

Stress has been documented to affect seroprevalence in wild-caught African black-footed penguins in South Africa. Higher anti - *Plasmodium* spp. antibody seroprevalence (38 and 62%) was reported in wild penguins held in captivity for rehabilitation after an oil spill, than in non-oiled birds (29 – 35%) (Graczyk et al., 1995a). A comparison of antibody levels in the population of Galapagos penguins over a long period of time will provide a better understanding of disease related fluctuations in the Galapagos penguins during stressful periods. Knowing the distribution of antibody levels in seropositive penguins during ENSO years could potentially determine if relapse infections increase during those periods.

There are a number of factors that would be expected to increase the Galapagos penguin's susceptibility to introduced diseases like *Plasmodium* including geographic isolation and a small total population size that is known to go through severe bottlenecks. Compared to other penguin species, the Galapagos penguin has lower genetic diversity when looking strictly at the average number of alleles per locus (3.0 alleles per locus) (see Nims et al., 2008). The level of gene flow, however, between smaller breeding colonies is high indicating that individuals move regularly throughout their range which increases the chance of introduced diseases to spread throughout their distribution (Nims et al., 2008). Until recently, the Galapagos penguins have been relatively disease – free, with all individuals reported by Travis et al. (2006) to be seronegative for all viruses tested, including West Nile Virus, indicating lack of exposure.

The Galapagos penguins also have very low MHC variability (three polymorphic sites and a nucleotide diversity of 0.01 in 157 base pair sequence of the exon 2 gene) as compared to other penguin species (see Bollmer et al., 2007). High variability of the MHC genes is important for the recognition of foreign antigens by an individual's immune system, especially to foreign pathogens, and a decrease in MHC variability might increase a species susceptibility to introduced diseases. It has been noted that some species with reduced numbers of MHC alleles may still be able to recognize a wide range of pathogens because the remaining alleles are often highly divergent (Hendrick et al., 2000). In the Galapagos penguins, the average amino acid divergence among alleles was only 4%, much lower than other species that also show very low MHC diversity (see Bollmer et al., 2007). With such low MHC variability in the Galapagos penguins, susceptibility to initial infection would likely be very high. However, what we see is high seroprevalence of anti – *Plasmodium* spp. antibodies suggesting they are surviving with the infections. Bollmer et al. (2007) propose that demographic factors like periodic bottlenecks may likely be a reason for the lack of MHC diversity. Given our results, it is possible that the cause of such low MHC variability in the Galapagos penguins might be due to a selective sweep through the population due to the introduction of the *Plasmodium* parasites. If so, the only individuals that survived initial infections are those that have the alleles for pathogen resistance, which would explain survival in the face of so many factors that would likely select against it. And if the introduction of *Plasmodium* coincided with a particularly strong ENSO event in the past, the combination of effects could have increased mortality in the population during that event.

For now, the penguins are able to cope and survive with this one introduced pathogen, but might not be so lucky in the presence of multiple pathogens.

High seroprevalence of malarial antibodies (23-91%) similar to what we report in this study has been determined in wild, Yellow-eyed penguins in New Zealand with higher seroprevalence at more northern sites where penguin populations overlap with higher concentrations of *Culex* spp. mosquitos (Graczyk et al. 1995c). The Galapagos archipelago sits on the Equator at latitude 0° 54' S, which is more northern than the yellow-eyed penguin range; therefore, it is not surprising that seroprevalence is higher in the Galapagos penguins at 97.2%. As the only tropical-breeding penguin, the Galapagos penguin is the only one found at latitudes where mosquitoes might be expected year - round. Sturrock and Tompkins (2007) failed to detect *Plasmodium* sp. DNA in wild, Yellow-eyed penguins (n = 143) from New Zealand and attribute the discrepancy of absolute lack of parasite DNA detection but serological results indicating high exposure to the parasite (Graczyk et al., 1995c), as being either an overestimation of exposure by serological tests, or that the initial infections occur in the penguins at an age that is poorly sampled for their study. High antibody levels to *Plasmodium* spp. have also been found in other endemic island avian species (Atkinson et al., 2001b; Jarvi et al., 2002; Woodworth et al., 2005) including wild populations of other penguin species (Graczyk et al., 1995a; Graczyk et al., 1995c).

Variations in anti – *Plasmodium* spp. antibody seroprevalence have been reported for wild and captive populations of six penguin species: African black-footed penguins (wild population = 52% seroprevalence), Adelie penguins (*Pygoscelis adeliae*) (0%), Gentoo penguins (*P. papua*) (33%), King penguins (*Aptenodytes patagonicus*) (58%),

little blue penguins (*Eudyptula minor*) (92%), captive Magellanic penguins (*S. magellanicus*) (43%), and captive yellow-eyed penguins (*Megadyptes antipodes*) (100%) (Graczyk et al., 1995b). Seroprevalence of *Plasmodium* spp. antibodies was higher in species that have overlapping distributions with a mosquito vector (*Culex spp.*) ranging from 33% in Gentoo penguins from French subantarctic territories to 100% in Yellow-eyed penguins from New Zealand (Graczyk et al., 1995b). Species with higher seroprevalence of anti - *Plasmodium* spp. antibodies are the more northern ranging penguins with potential parasite exposure throughout the year, whereas species with lower seroprevalence may only be exposed to competent *Plasmodium* sp. vectors during migration, allowing some penguins to remain unexposed (Graczyk et al., 1995b). Our results show seroprevalence in the Galapagos penguins, the most northern ranging penguin species, to be consistent with this trend at 97.2%.

The suspected *Plasmodium* vector on the Galapagos archipelago is the mosquito species *Culex quinquefasciatus* which was first documented on the Galapagos Islands in the 1980s (Peck et al., 1998) and has since been well established (Whiteman et al., 2005). *Culex quinquefasciatus* is a known *Plasmodium* vector elsewhere (Atkinson et al., 2000). The range of this fresh water breeding mosquito on the archipelago is limited since there are very few fresh water sources outside of human inhabited areas. The only recorded native species of mosquito on the islands that are known to bite birds, *Aedes taeniorhynchus*, may also be a potential vector of *Plasmodium*. Given that *A. taeniorhynchus* breeds in brackish water, is known to take blood meals from birds, and that some of the penguins testing positive for *Plasmodium* infection were sampled in areas lacking regular fresh water sources, this native species cannot be discounted as a

possible vector. Therefore, it is important to determine not only the vector, but also which bird species on the island is acting as a reservoir for the parasite. If the penguins are not competent hosts for the parasite, there must be another species, or multiple species, on the archipelago acting as the reservoir for the transmission of *Plasmodium*.

Recapture data of seropositive penguins and very high anti – *Plasmodium* spp. seroprevalence in both 2008 – 09 (97.2 %) and 2004 – 05 (96.8 %) indicates that the Galapagos penguins are surviving in the presence of *Plasmodium* parasites, at least under normal environmental conditions. Our results also give strong support to the idea that more than one detection method should be used to determine the extent of *Plasmodium* in populations that may not be competent hosts for the parasite. Similarly, even in populations that are capable of transmitting the malaria parasite, multiple detection methods are necessary given that both blood smear and PCR techniques can underestimate the extent of *Plasmodium* infections (Jarvi et al., 2002). And finally, understanding the transmission dynamic (e.g. reservoir species and vector species) of this parasite on the archipelago is essential for the long - term conservation of the Galapagos penguin and other susceptible endemic avian species. If it is shown that either the mosquito vector or reservoir species for *Plasmodium* transmission is a non - native species, steps can be implemented for their eradication.

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FIGURES

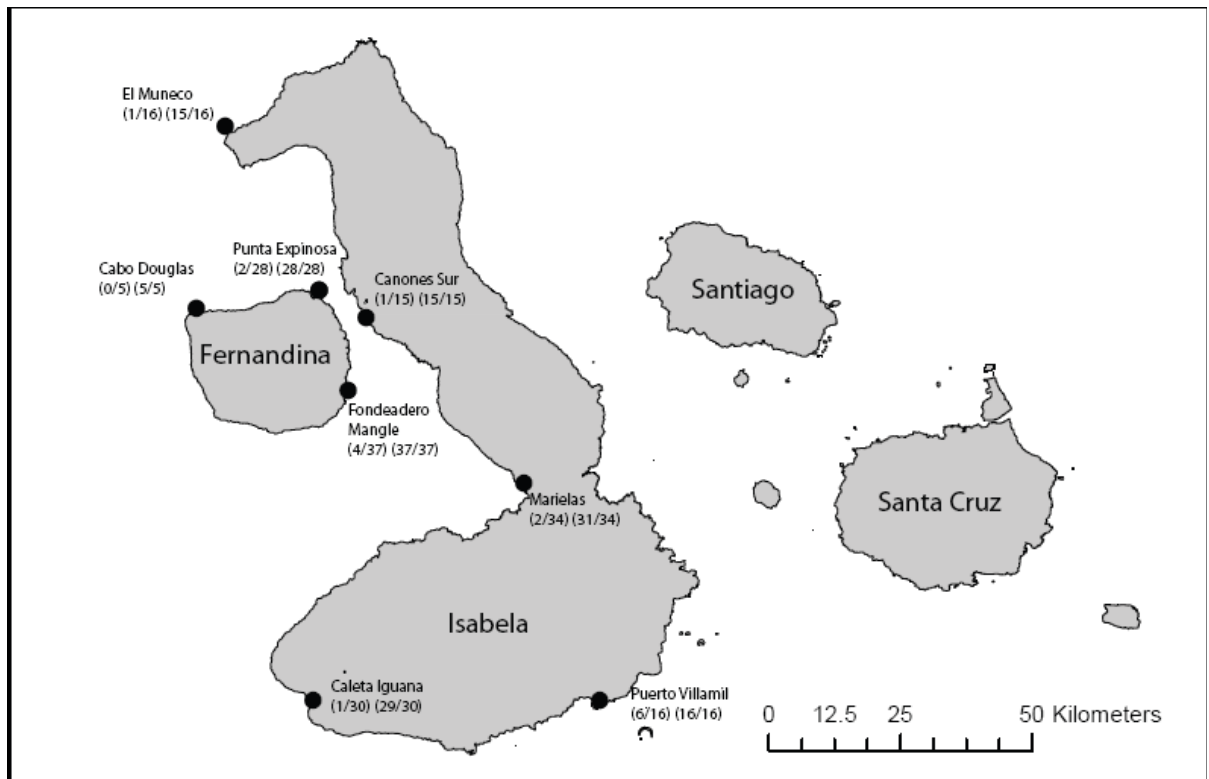


Figure 1. Map of 2008 – 2009 sample sites. Total of eight sites located on Isabela and Fernandina islands. Numbers in parentheses show prevalence of parasite DNA by PCR and Seroprevalence (no. PCR positive/total) (no. seropositive/total).

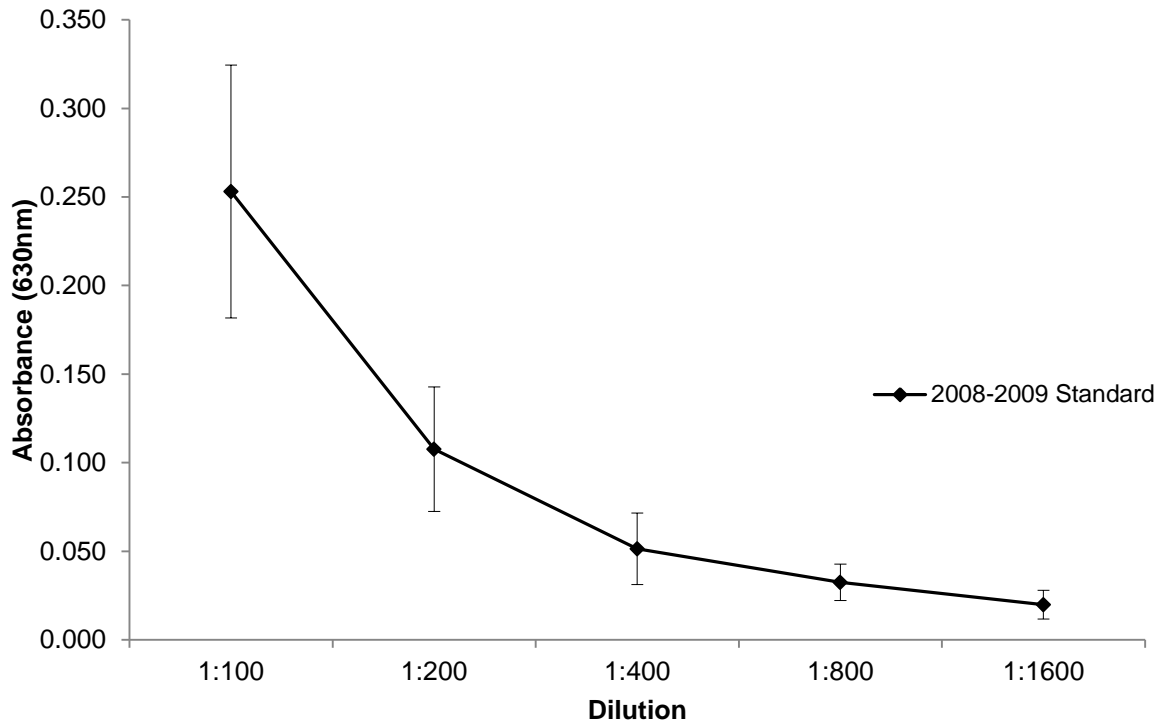


Figure 2. Standard: pool of 3 penguins from the 2008 field season that amplify as *Plasmodium* on PCR, run in serial dilution from 1/100 to 1/1600 on each plate. Curve includes the mean absorbance values across 10 plates \pm SD.

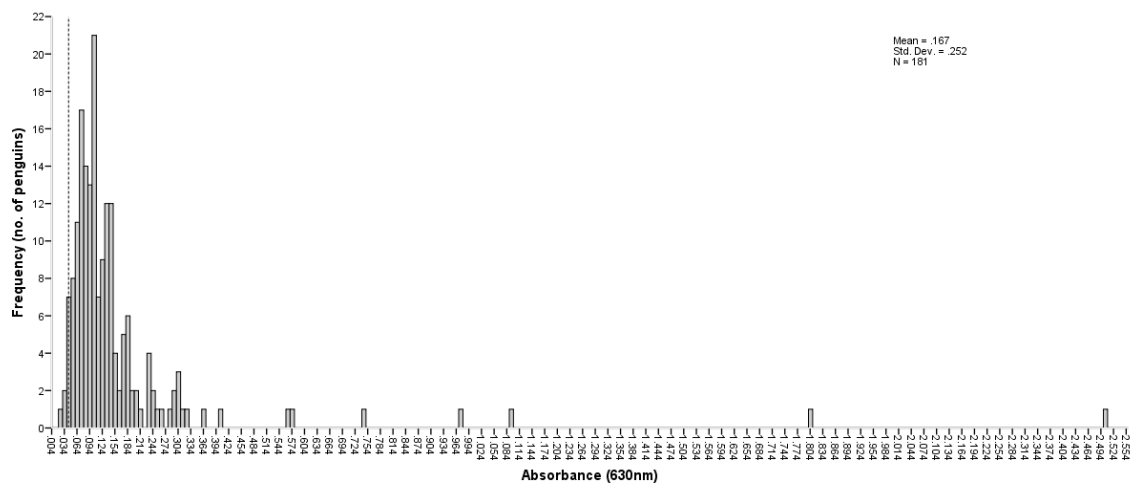


Figure 3. Frequency distribution of antibody levels, as shown by absorbance (630 nm) of 181 total penguins, 149 adults, 24 juveniles and 8 penguins of undetermined age. Positive cut-off indicated by dotted line at an absorbance of 0.044.

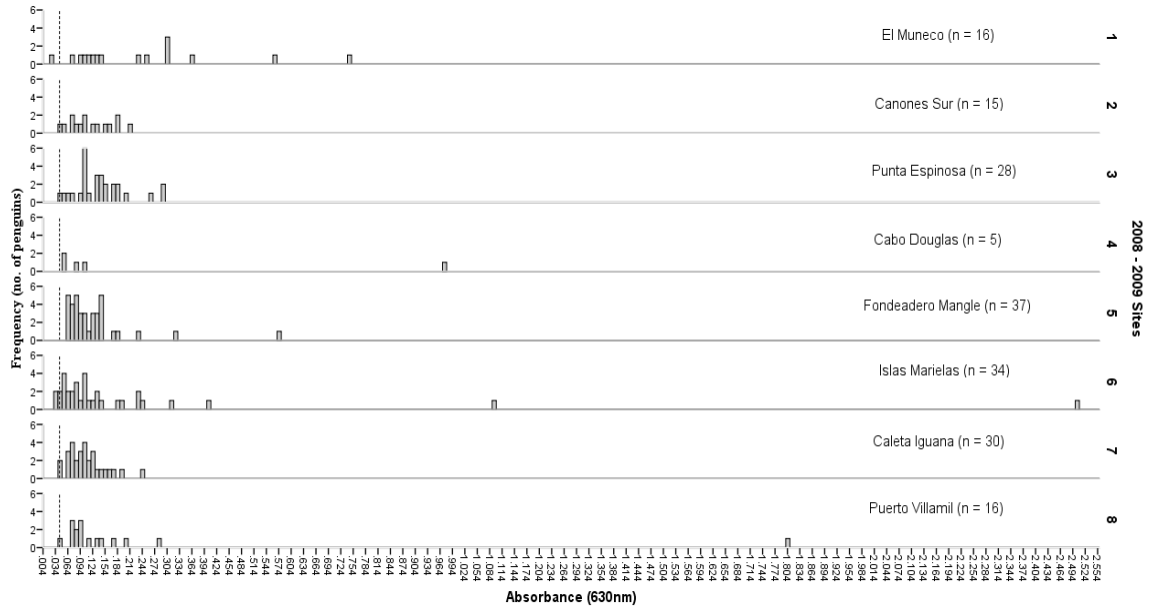


Figure 4. Frequency distributions of antibody levels, as shown by absorbance (630 nm), for all eight sites sampled in 2008-2009. Sites are listed in order from north to south. Positive cut-off indicated by dotted line at an absorbance of 0.044.

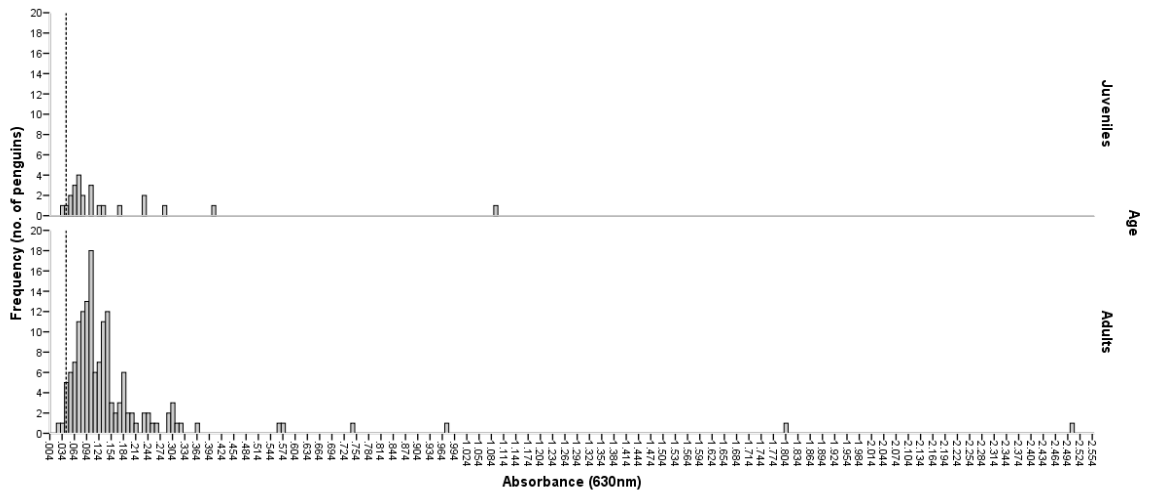


Figure 5. Frequency distributions of antibody levels, as shown by absorbance (630 nm), of 2008 - 2009 penguin samples by age. Juveniles (n = 24), Adults (n = 149). Positive cut-off indicated by dotted line at an absorbance of 0.044.

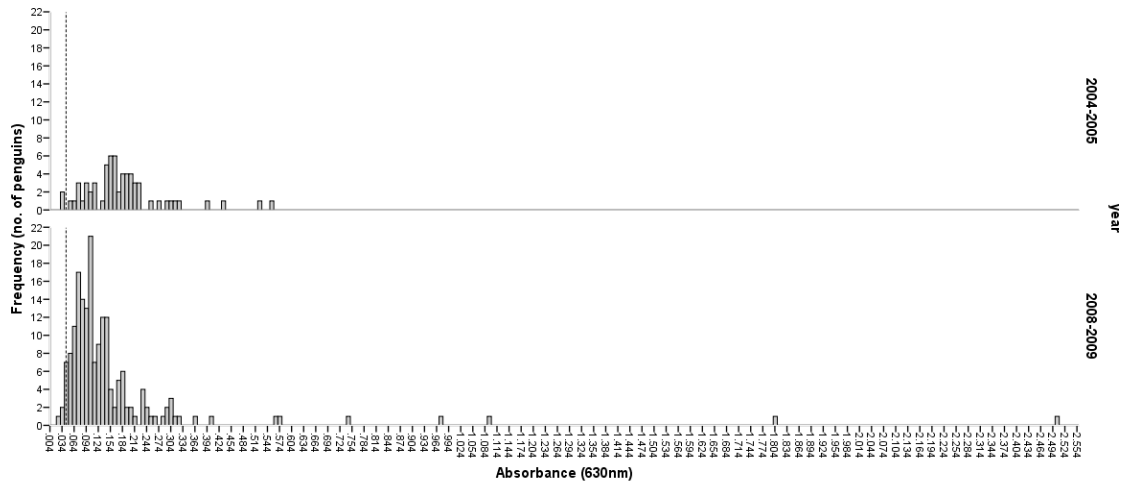


Figure 6. Frequency distribution of antibody levels, as shown by absorbance (630nm), of 64 penguins from the 2004-2005 field seasons as compared to 181 penguins from 2008 – 2009 field seasons. Positive cut-off indicated by dotted line at an absorbance of 0.044.

TABLES

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
Canones 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	.025 - 2.503

Table 1. Seroprevalence of anti-*Plasmodium* immunoglobulins in wild, 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 1:1600 dilution.

Year		n	Mean	Median	SD	IQR
2008 – 09	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 – 05	Adult	64	0.184	0.166	0.101	0.09

Table 2. Descriptive statistics for antibody distributions, as shown by absorbance, of all individuals tested, separated by year and age class.

Recapture comparisons		Absorbance values			Difference (%)
Site	Penguin ID	2004-2005	2008	2009	
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

Table 3. Absorbance value comparisons of individuals sampled in more than one year. 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 1:1600 dilution. *Highlighted values indicate individuals that tested as positive by PCR.

2008-2009		No. of PCR positive penguins				
Site	Total no. of penguins	Waldenström only	Perkins & Schall only	Both	Total no. positive	(%)
El Muneco	16	1	0	0	1	6.3
Punta Espinosa	28	1	0	1	2	7.1
Cabo Douglas	5	0	0	0	0	0.0
Canones Sur	15	0	0	1	1	6.7
Fondeadero Mangle	37	2	1	1	4	10.8
Islas Marielas	34	0	1	1	2	5.9
Caleta Iguana	30	1	0	0	1	3.3
Puerto Villamil	16	1	1	4	6	37.5
Total	181	6	3	8	17	9.4

Table 4. Total number of penguins that tested positive for the presence of *Plasmodium* parasites by PCR. Total number of individuals that tested positive with the following PCR primers: Waldenström primers only, Perkins & Schall primers only and individuals that tested positive on both methods.