Prevalence and drivers of blood parasitism in African penguins (*Spheniscus demersus*)

By

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ABSTRACT

In the past decade African penguin (*Spheniscus demersus*) populations have experienced declining population sizes of > 60% in some instances. This has primarily been attributed to shifting prey availability and competition with regional purse-seine fisheries; however, possible novel threats exacerbated by diminishing population sizes and increased stress, may also be important contributors to the regional persistence of the species. These threats include the impacts of arthropod-borne blood parasites on the health of penguin populations. While parasitism of wild penguins has been poorly studied, susceptibility to infection with blood protozoa is well established in a wide range of penguin species held in captivity. This thesis assesses the prevalence of blood parasites in wild African penguins throughout the greater Agulhas-Benguela ecosystem. Using PCR-based techniques, 317 individuals were screened for the presence of known haemoparasite species of *Plasmodium*, *Haemoproteus* and *Babesia* across 12 breeding colonies. *Babesia* infection was confirmed for 60% of wild birds sampled, but methods used did not indicate infection with either *Haemoproteus* or *Plasmodium* species. Generalised linear modelling of ecological and life history parameters suggests that *Babesia* prevalence is primarily driven by a colony's distance from the mainland, decreasing significantly as distance from the mainland increases. Captive birds held at the SANCOBB rehabilitation facility in Cape Town present with both *Plasmodium* and *Babesia* species, providing the positive controls for the study. The relative scarcity of *Plasmodium* and *Haemoproteus* infection in the wild may be the result of an absence of suitable vectors and/or high post mortality in the host. Alternatively, the sensitivity of the method used requires further investigation as *Plasmodium* infection has been confirmed previously in penguin populations using PCR-based approaches. This study provides the first baseline estimate of blood parasitism in African penguins across their breeding range, and raises the need for further research and monitoring. The results suggest that high *Babesia* prevalence in African penguins may be related to (1) an ecological system of chronic-exposure to infection reservoirs, such as the co-occurring cormorant and gannet populations and (2) increasing anthropogenic impacts, especially in mainland colonies. Data on blood parasitism in co-occurring seabird species is required to fully elucidate their role in *Babesia* infection dynamics in the region. To improve understanding and facilitate timely detection of changes in blood parasite exposure, standardised methodologies are advocated to better inform the conservation management of this iconic species.

Keywords: seabirds, haemoparasites, *Babesia*, GLMMs, distance from mainland, infection reservoirs, anthropogenic impacts

1. INTRODUCTION

1.1 19th and 20th century decline in African Penguins

African penguins (*Spheniscus demersus*) are endemic to the greater Agulhas–Benguela upwelling ecosystem of southern Africa. Historically, these penguins were harvested in large numbers for food, as a fuel source in ship boilers and for their fat [1]. Egg collecting was also common, with up to 48% of the total number of eggs on a breeding island removed as a food source, causing large-scale population declines [2]. Across their distribution, breeding is confined to 28 colonies occurring between Hollamsbird Island, Namibia and Bird Island, South Africa. These are broadly grouped into three breeding regions, with 11 breeding colonies in Namibia, 11 in the Western Cape and six in the Eastern Cape of South Africa [3].

The typical non-breeding range falls between 18° S on the Namibian coast and 29° S on the coast of KwaZulu-Natal, an expanse of ca. 3200 km of coastline. Vagrant birds have however been recorded as far north as Sette Cama (2°32' S), Gabon on the West African coast [4], and north-east to the Limpopo River mouth (25° S) and to Mozambique on the African east coast. Generally, this species occurs within 20 km of the coast [5], though it has been recorded foraging up to 100 km offshore [6]. This is a more common occurrence on the Agulhas Bank, where the distribution of their prey species extends further offshore [7]. The collapse and continued decline of this iconic seabird has recently heralded its reclassification by the International Union for the Conservation of Nature (IUCN) from 'Vulnerable' to 'Endangered', as just seven islands now support ca. 80% of the remaining global population [8]. Though current legislation protects the species from ongoing exploitation, the population has recently sustained a decline of ca. 35 000 breeding pairs (>60%) in the past decade [9] and there is considerable debate on the primary drivers. Together, historic and contemporary factors have reduced the breeding population of African penguins by approximately 95% [10].

1.2 Modern threats to African penguins

While the current decline of African penguins has primarily been attributed to food abundance, food distribution and its quality [9], there is increasing concern surrounding novel threats faced or exacerbated by diminishing population size and increased stress. Predation is an important stress to African penguins. Adults are preyed upon by great white sharks, Cape fur seals; and according to isolated reports, killer whales, caracals, leopards, feral cats and domestic dogs. Furthermore, chicks and eggs fall prey to: mole snakes, kelp gulls, white pelicans, mongooses, genets and Cape clawless

otters. Additional threats include: intra- and interspecific competition for food and breeding sites, climatic variation, habitat degradation, pollution, disease and human disturbance [11,12] . With respect to disease, an important but understudied threat to the health and persistence of wild African penguin populations is that of infection by protozoan blood pathogens. Infection with these haemoglobin-feeding parasites results in a lowered red blood cell (RBC) count. This acute and chronic anaemia has been shown to cause increased morbidity and even mortality in penguins, particularly when under stress [13].

1.3 Blood-parasitism in birds

The majority of avian families studied to date are susceptible to infection by blood parasite, known as haemoparasites in the wild [14]. In the tropics, bird species are particularly prone to infection, with very high prevalence reported (> 30% of families) [15]. Species may play host to a suite of blood-inhabiting protozoa and nematode worms, transmitted by haematophagous arthropod vectors [13]. Nematode worms known to infect birds include those from the family Filariidae and Haemoflagellates in the genus *Trypanosoma* [13], while intra-cellular blood parasites include: Haemosporidia of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon*, Heamogregarinidae of the genus *Hepatozoon* and Piroplasmida of the genus *Babesia* [16]. These haemoparasites are responsible for a number of diseases in wild and domestic birds, including avian malaria and their negative effects on fitness in wild bird populations have been well established [17]. Blood parasites regulate host populations by exerting a variety of important selection pressures on: survival [18–20], reproductive success [21–25], and even plumage colouration [26,27]. Thus, at high prevalence, haemoparasite infections may have important ecological and evolutionary consequences [16], through changes in host community structure [28].

While blood parasites are common in many wild bird populations, a number of avian groups are reportedly less susceptible e.g. swifts [29], waders [30], parrots [31] and seabirds [16,32–35]. Seabirds in particular, are often reported as being free of infection, even in the presence of potential vectors [16]. Although the rates of infection vary greatly across seabirds [36,37]: *Haemoproteus* parasites are reported to be common in frigatebirds and gulls, while *Hepatozoon* parasites are found mainly in albatrosses and storm petrels, and *Plasmodium* has been reported mainly in penguins [16]. While the nematode worms from the family Filariidae occur in many species of birds, they have not been reported in penguins [13]. The ecological and evolutionary significance for this wide taxonomic

variation in parasite prevalence remains poorly understood [16,38,39]. Studies on wild bird populations suggest that blood parasites are less common in certain habitats such as the Arctic tundra [40], arid- [41,42], island- [43] and marine-environments [44–46]. A recent review by Quillfeldt *et al.* (2011) confirmed that blood parasites were absent from all Antarctic and Arctic seabirds studied, suggesting that infection occurrence increased towards milder climates, mirroring a lack of suitable vectors [33,47]. Furthermore, it was suggested that latitudinal gradients in the prevalence of blood parasites have been found in other bird species [48] and even within species patterns [49]. In lieu of these trends, a series of hypotheses have been proposed to explain their absence or prevalence gradients [50], such as the absence or scarcity of suitable vectors, highly specific associations between host and parasites with infrequent host switching (host-parasite assemblage), host immunological capabilities preventing infection by parasites, and competitive exclusion of blood parasite vectors mediated by ectoparasites [16].

1.4 Dynamics of haematozoan infection

Blood-parasitism in African penguins is particularly interesting as this species breeds at relatively low latitudes (Namibia) and in temperate environments (Eastern Cape), and comes into contact with both a number of potential arthropod vectors (e.g. black flies, mosquitoes, louse flies, biting midges, ticks, fleas etc.) and a number of sympatric bird species (e.g. cormorants, gannets, kelp gulls etc.), which may serve as a reservoir of infection [13]. This suggests a complex ecological interaction between host, parasite and vectors, influenced by a variety of factors. These may include the presence of suitable vectors and compatible parasites within the ecosystem, the feeding preferences of vectors, the population density of hosts, and opportunities for vectors to feed [13]. In addition, parasites show varying degrees of host specificity and feeding preferences, for both their vertebrate hosts and invertebrate vectors.

Ultimately, the availability of suitable arthropods within the range and habitat of the vertebrate hosts, the numbers and life span of both vertebrate hosts and invertebrate vectors, and the presence or absence of other reservoirs or host species can affect the probability of infection (Figure 1), as both the arthropod vector and vertebrate host play integral roles in the parasites' life cycles [13].

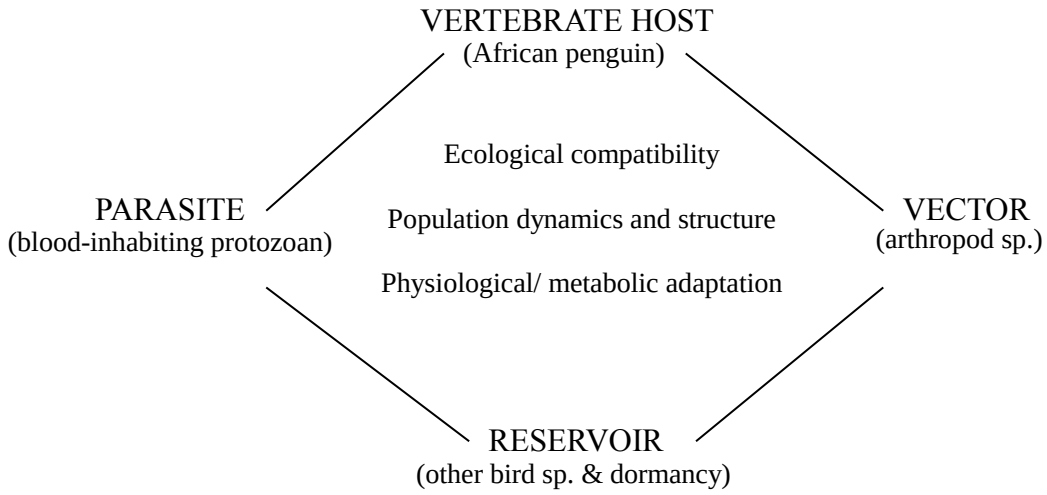

Figure 1 A schematic of the potential interactions between hosts, reservoir hosts, haemoparasites and their vectors, adapted from Jones and Shellam (1999).

The distribution, life cycles and environmental adaptability of these vectors often dictate the range, prevalence and reoccurrence of the haemoparasites they are thought to carry. Thus they play a pivotal role in interpreting any haemoparasitic distribution. In captivity, African penguins present with infection for the malarial agents: *Plasmodium relictum, P. elongatum, P. juxtanucleare* or *P. cathermerium* [14], together with *Leucocytozoon tawaki* [51] and *Babesia* sp. [52].

Haemoproteus however, despite being the most widespread genus of avian blood protozoan, has never been reported in penguins, neither in the wild nor in captivity [53]. Whilst few arthropod vectors have been confirmed for wild African penguins, many have been established for captive species, such as *Plasmodium* spp., which are known to be transmitted by *Aedes* and *Culex* mosquitos and *Leucocytozoon* spp., transmitted by simuliid blackflies and ceratopogonid midges (*Culicoides* sp.) [13]. Whilst it is unknown whether the *Ixodes uriae* tick is a competent host or vector to any of the blood parasites recorded thus far in, it is thought to play a role in the transmission of *Babesia peircei* and *Borrelia* sp. to penguins ([54,55]) and between penguins and migrating birds such as skuas and storm petrels [56] .

Infection with these haemoparasites ultimately results in a lowered red blood cell count RBC count. This acute and chronic form of anaemia has been shown to cause increased morbidity and even mortality in penguins, particularly when under stress [13]. These pressures are further exacerbated by the fact that African penguins are primarily anaerobic hunters, requiring a high RBC count to replenish oxygen-depleted tissue. Such a hindrance could lead to exhaustion or increased predation.

Furthermore, sublethal levels of haemoparasitic infection may have effects on: reproductive fitness, clutch size, quality and frequency of moult, nestling development, fledgling periods, growth rates, moulting and predatory pressure [57]. Without adequate data, the extent of the risk posed by introduced or exacerbated haemoparasitic infections to penguin populations as a result of environmental and other changes, is not known. Several possible factors, directly or indirectly anthropogenic, which have the potential to produce or increase exposure to, or decrease resistance to exotic pathogens, should thus be considered in the conservation and biodiversity management plan [11] for this endangered species.

1.5 Aims and Hypotheses

Research into the role of pathogens in host ecology and species conservation has grown in recent decades and the advent of Polymerase-Chain-Reaction (PCR) based methods now allows greater insight into spatial patterns of infection as well as pathogen diversity. Using a combination of PCR-based screening of populations together with an analysis of ecological, geographic and life history parameters, this study aims to: (1) characterise blood pathogen prevalence across the breeding range of African penguins and (2) determine some of the likely drivers of pathogen prevalence and their potential importance in conservation programmes.

Patterns of pathogen prevalence are both directly and indirectly influenced by a complex interaction of factors between the parasite, arthropod vector and host. Additionally there is invariably an effect of scale and seasonal variability on this distribution. To assess the relative contributions of both biotic and abiotic factors in the prevalence of penguin haemoparasites, a multi-scale model framework was used to rank the investigated drivers of pathogen prevalence. Separate hypotheses were formulated for these geographic, colony and individual scale drivers:

1. At the geographic scale, latitude, longitude, breeding colony (nested within each breeding region) and the interaction of these effects were considered. Given their varied histories of exploitation, protection and exposure to anthropogenic disturbances, colonies were not predicted to experience a shared single driver of pathogen prevalence.

2. For each colony, the effects of distance from the mainland (km), population size (numbers of breeding pairs) and levels of heterozygosity measured at neutral satellite markers, as well as interactions between these were considered. Parasite prevalence was predicted to decrease with distance from the mainland because of reduced exposure to potential vectors. In addition, prevalence was predicted to increase with colony size as density dependent transmission increases, but decrease with increasing heterozygosity. Whilst microsatellite markers are non-coding neutral markers, they have been used extensively as a general measure of genome variation.

3. At both the colony and geographic scale, the effects of these variables, as well as their interaction with sex and life stage of individuals were also considered. For all scenarios, females were predicted to be more heavily parasitized. Females are slightly smaller than males and coupled with their increased foraging effort in the breeding season, this may place them at greater risk of reduced body condition and thereby infection [58]. It was also expected that, as chicks are unable to preen themselves effectively for ecto-parasites, they are likely to experience increased exposure to vectors and so haemoparasitism.

2. MATERIALS & METHODS

2.1 Ethics statement

Research reported here was carried out on African Penguin blood and feather samples collected by Dr Lisa Nupen as a component of her PhD thesis [59]. These were collected in accordance with research and ethics permits approved for the collection of samples (University of Cape Town Science Faculty Animal Ethics clearance number: 2009/v21/LN; Oceans and Coasts branch of the South African Department of Environmental Affairs permit number: RES2010/66; Cape Nature (Western Cape Nature Conservation Board) permit number: AAA-004-00520-0035; SANParks permits were approved, but no permit number was supplied). Further permits were obtained from the Namibian Ministry of Environment, Natural Resources and Transport (Wildlife Enforcement and Permits Division), Wildlife Trade and Conservation Section (no permit number supplied) to collect blood samples from Ichaboe and Mercury Islands; however, these only permitted sampling of African Penguin chicks. Samples from Possession Island were collected by Dr Nola Parsons as part of the SANCOBB bird health survey in 2010.

2.2 Sampling

Sample collection

A total of 265 blood and 52 feather samples were taken from African penguin breeding adults or chicks at 12 colonies located throughout their range in the Agulhas-Benguela ecosystem (Figure 2), between January 2009 and March 2011. The colonies sampled are grouped geographically into three broad breeding regions: Namibia (Ichaboe Island, Mercury Island, Halifax Island and Possession Island), the Western Cape, South Africa (Stony Point, Jutten Island, Boulders Beach, Robben Island, Dyer Island and Dassen Island) and the Eastern Cape, South Africa (Bird Island and St. Croix Island). Details of individuals sampled at each colony are provided in Table A1 of the Appendix. The colonies sampled support the vast majority of the African penguin population and were selected because they were the largest colonies i.e. they represent all African penguin colonies which boast >100 breeding pairs, with the exception of Vondeling Island in the Western Cape and Seal Island in the Eastern Cape, which were not sampled in this study. All other African penguin breeding colonies support fewer than 100 breeding pairs.

Study area

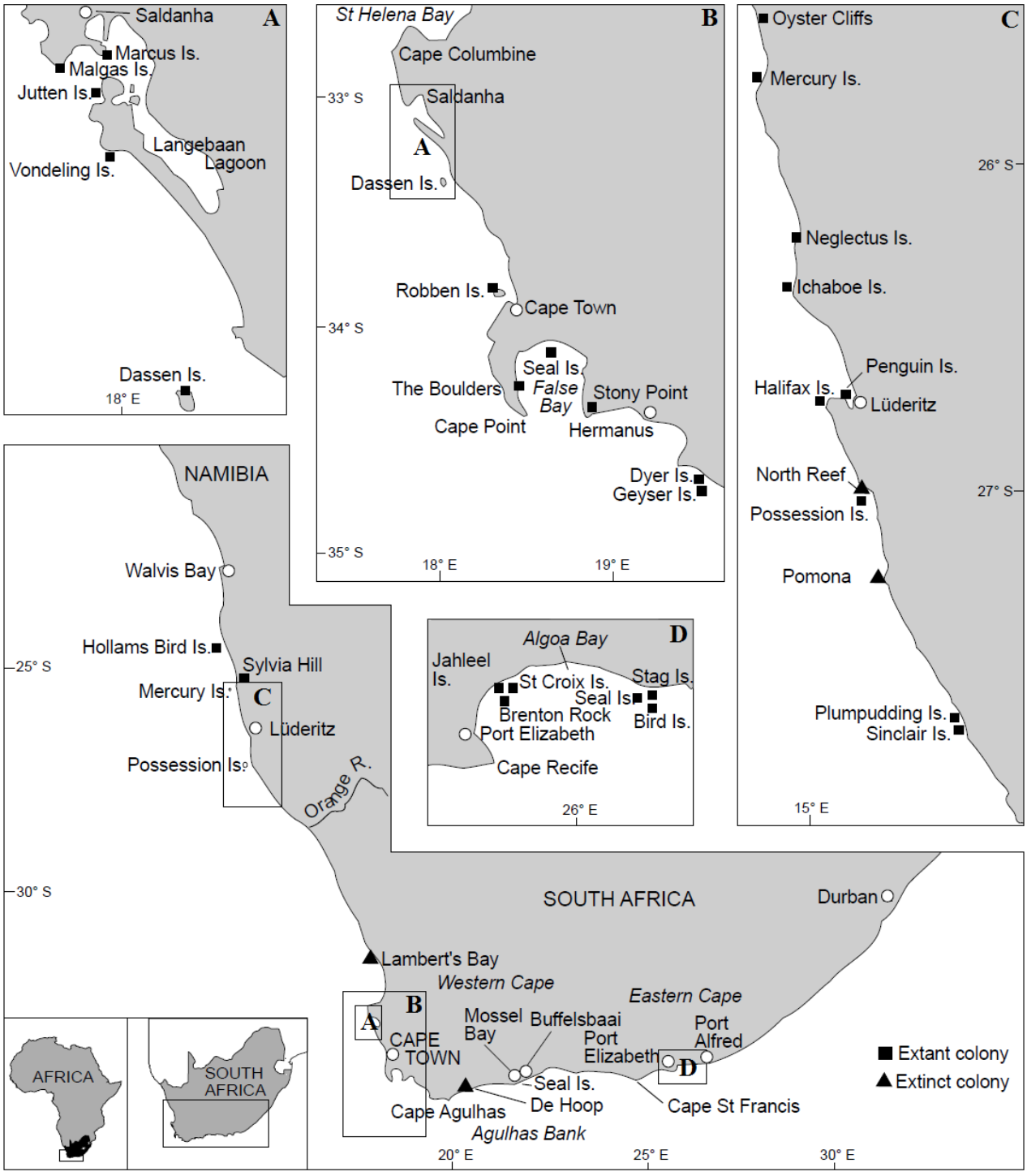

Figure 2 Breeding localities and geographic distribution of African penguins (1990-2010). Different symbols are used to depict localities where breeding took place, or was believed to take place, in 2010 (solid squares) and those where breeding ceased after 1990 (solid triangles). The three breeding regions are depicted by plates 'A' and 'B' (Western Cape), 'C' (Namibia) and 'D' (Eastern Cape).

2.3 Molecular analyses

Sample preservation & storage

Blood samples were collected into Longmire's Lysis buffer solution and feather samples were stored in ethanol (99.9%). All samples are archived at the FitzPatrick Institute of African Ornithology at the University of Cape Town.

DNA extraction

Total genomic DNA was extracted from the original samples using DNeasy© Blood & Tissue Kits (Qiagen, Valencia, CA), according to the manufacturer's guidelines. The genomic DNA extraction protocol was modified for feather samples with an increased incubation step extended to 24 hours. Where re-extraction was required, a standard salt-extraction procedure was used. Briefly, blood samples were air dried (in the case of 99.9% ethanol storage, this was aspirated off), before suspension in 1ml of Longmire's Lysis buffer solution. After adding 20µl of Proteinase K (20µg/µl), samples were left to digest for either two days at 37°C (feathers) or for 30 minutes at 55°C (blood). Following this, ½ the volume of 5M NaCl was added and shaken for 20 minutes, then a whole volume of 24:1 chloroform: isoamyl alcohol was then added, vortexed and shaken for 10 minutes. The samples were spun down at 13 000 rpm for two minutes, the supernatant was removed and stored in 600µl isopropanol at -80°C, either for 30 minutes or overnight. Once precipitated, the samples were spun down at 12 000 rpm for 10 minutes and the supernatant removed, the pellet was washed with 70% EtOH (ice-cold), before being removed and air-dried. The pellet was then dissolved in 50µl TE buffer solution. These extraction stocks were then stored at -80°C (long-term) or - 4°C (short-term).

Control validation through microscope analysis

Positive infection controls were obtained from SANCOBB, either from live individuals in rehabilitation or dead birds which had died in captivity or been brought to the centre. Individuals were examined pre- and/ or post-mortem and their sex determined. Blood samples were also used as positive controls for known male and female birds. For parasite screening (positive controls only), blood smears were prepared and air-dried, fixed with methanol, and later stained with Giemsa stain (1/10 *v/v*) for 30min. Blood smears from positive control birds were scanned for parasite infections using a compound microscope (Nikon *Eclipse* 50i Compound Microscope) . These were analysed at 100× under oil amplification following the methods described in Merino et al. (1997). Photomicrographs of both *Plasmodium* and *Babesia* positive infections were taken.

Molecular sex determination of penguin samples

All samples were sexed following the PCR-based methods developed by Fridolfsson and Ellengren [60]. Female birds are heterogametic (ZW) while males are homogametic (ZZ), and sexing can thus be done by the detection of the W chromosome or W chromosomal sequences in a sample of unknown sex. This method for molecular sexing on non-ratite birds is based on the detection of a constant size difference between chromosome-helicase-DNA binding protein for CHD1W and CHD1Z introns. Each 10µl reaction contained: 5µl Thermoscientific^{TM} DreamTaq^{TM} Green PCR Master Mix (2X) 200, 1.2µl DNA at [0.1mg ml^{-1}], 3µl ddH_2O and [0.1 pmol ml^{-1}] of primers 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3'). The final $MgCl_2$ concentration was optimised to 1.75mM. PCR's were performed on Applied Biosystems 2720 and Veriti® 96-Well Thermal Cyclers. The thermal profile comprised of an initial denaturing step of 95°C for 5min, followed by 40 cycles of a 95°C denaturation step for 30sec, a 53°C annealing step for 45sec and a 72°C extension step for 50sec, the final extension step was 72°C for 10min before holding at 4°C (Table A2). PCR products were separated in 2% agarose gel electrophoresis (SeaKem® LE Agarose, Whitehead Scientific (Pty) Ltd). Males were recognised in gel electrophoresis as displaying a single PCR product (from CHD1Z), while females showed two different products (from CHD1W and CHD1Z), approximately 100 bp apart (Figure).

Diagnosing Babesia infection using PCR-based methods

All samples were screened for *Babesia* infection following the PCR-based methods developed by Quillfeldt *et al.* [61]. PCR diagnosis of *Babesia* is based on a highly conserved target region (797bp) within 18s ribosomal DNA of this haemoparasite. Only in the presence of the parasite will these specific primers amplify out this region, thus acting as a proxy for the presence of infection. Each 10µl reaction contained: 5µl Thermoscientific^{TM} DreamTaq^{TM} Green PCR Master Mix (2X) 200, 1.2µl DNA at [1 mg ml^{-1}], 3µl ddH_2O and [0.1 pmol ml^{-1}] of primers Bab600F (5'-TCGTAGTTGAACTTCTGCTG-3') and IsospR (5'-ATTGCCTCAAACTTCCTTGC-3'). The final $MgCl_2$ concentration was optimised to 1.75mM. PCR's were performed on Applied Biosystems 2720 and Veriti® 96-Well Thermal Cyclers. The thermal profile comprised of an initial denaturing step of 95°C for 5min, followed by 40 cycles of a 95°C denaturation step for 30sec, a 58°C annealing step for 30sec and a 72°C extension step for 60sec. The final extension step was 72°C for 10min before holding at 4°C (Table A2). PCR products were separated by gel electrophoresis on a 2% agarose gel

(SeaKem® LE Agarose, Whitehead Scientific (Pty) Ltd). Infected individuals were recognised in the gel as displaying a single PCR product band of ca. 797 bp in length (Figure A2).

Nested diagnosis of Haemoproteus and Plasmodium infection

All samples were screened for either *Haemoproteus* or *Plasmodium* infection following the nested PCR-based methods of Hellgren *et al.* (2004)[62]. Nested PCR-based diagnosis of these haemoparasites is based on a highly conserved target region (580bp) within *Cytochrome b* region of the haemoparasite mtDNA. Only in the presence of the parasite will these specific primers amplify out this region, thus acting as a proxy for the presence of infection. Each 10µl reaction contained: 5µl ThermoscientificTM DreamTaqTM Green PCR Master Mix (2X) 200, 1.2µl DNA at [1mg ml^{-1}], 3µl ddH2O and 0.1pmol/ml of primers HAEMF (5'- CATATATTAAGAGAAT-TATGGAG-3') and HAEMNR2 (5'- AGAGGTGTAGCATATC TATCT-AC-3') at [50pmol ml^{-1}]. The final MgCl2 concentration was optimised to 1.75mM. PCR's were performed on Applied Biosystems 2720 and Veriti® 96-Well Thermal Cyclers. The thermal profile comprised of an initial denaturing step of 95°C for 5min, followed by 40 cycles of a 95°C denaturation step for 30sec, a 50°C annealing step for 30sec and a 72°C extension step for 45sec. The final extension step was 72°C for 10min before holding at 4°C (Table A2). PCR products were separated on a 2% agarose gel (SeaKem® LE Agarose, Whitehead Scientific (Pty) Ltd). Infected individuals were recognised the gel as displaying a single PCR product band of ca. 580 bp in length (Figure A3).

DNA sequencing, alignment & phylogenetic analysis of Plasmodium positive controls

The PCR primers used in this study are all highly specific to their targets and have been verified to only amplify the target gene of the parasite species of interest [60–62]. Nevertheless, the lack of PCR results indicating positive infection for *Plasmodium* and/ or *Haemoproteus* was of concern. To double-check the PCR, positive control samples were sequenced on an ABI300 Genetic Analyser (University of Stellenbosch, Central Analytical Facilities, SA). The resulting sequences were then compared to known parasite strains using DNA sequence databases available at Genbank® (http://www.ncbi.nlm.nih.gov/genbank) and the avian malaria database MalAvi (http://mbio-serv2.mbioekol.lu.se/Malavi/). Searches for matching sequences were carried out using BLAST (Basic Local Alignment Search Tool) and a selection of sequences with a positive match to the study controls were then used to create a DNA sequence alignment using the ClustalW Multiple Alignment

[63] accessory application in BioEdit® Sequence Alignment Editor V7.0.9.0 [64]. Following Bensch et al. (2000) [65] the phylogenetic placement of the penguin positive control was then estimated using the neighbour-joining method in MEGA v6.0 [66]. The tree was obtained using amino acid sequence differences and node support was tested using 1000 bootstrap replicates. The tree was rooted using the closely related *Haemoproteus lanii*.

2.4 Statistical analysis

Haemoparasitic prevalence

Pathogen prevalence was calculated for each colony and for all colonies sampled within the three breeding regions for African penguins. All subsequent analysis was carried in SPSS (version 21, IBM, USA). A Kolmogorov-Smirnoff test for normality and Levene's test for homogeneity of variances for all grouping levels was first applied to ensure the data met the assumptions of subsequent analyses. To quantify variation in infection within and between colonies, the infection prevalence (%) was calculated for each population:

$$\frac{Number\ of\ infected\ individuals}{Total\ number\ of\ individuals\ sampled} \times 100$$

Modelling the drivers of haemoparasitic prevelance using a general linear mixed model framework (GLMM's)

Various intrinsic (e.g. sex and life stage) and extrinsic (e.g. latitude, breeding region, colony, distance from mainland, colony size and observed heterozygosity) factors can influence the distribution of pathogen prevalence across a species range. To tease apart their relative effects on infection prevalence, a hierarchically structured model was used to reflect the ecological scales over which pathogen prevalence is influenced in natural populations: the geographic, colony and individual scale.

A model selection approach based on Akaike information criterion (AIC) was used to determine which model, out of a range of candidate models, best explained the pathogen prevalence throughout the range of African penguins at each of the three ecological scales (97,98). The model with the lowest AIC value was considered the most parsimonious and the difference in AIC scores (Δ_i) were calculated to determine the likelihood that a given model was the best model relative to other candidate models (98). A Δ_i value of zero indicates the best fit model; values up to two indicate

models with substantial empirical support; those between four and seven indicate less support and those with values >10 have essentially no support (98). Akaike weights (w_i) were also used to calculate the probability that a given model was the best among a candidate set [67], together with the evidence ratio (ER) which provides a measure of how much more likely the best model is than the next [68]. Thus the best fit model has the lowest Δ_i and ER, whilst having the highest w_i value (98). Once the best fit model was identified for each scale, the effects of each scale on pathogen prevalence were identified, using regression analysis and the Wald's Chi-squared test of association. In order to determine the importance of each variable included in the best model for each scale, the summed Akaike weight (w_+) for all models containing that particular variable was calculated. The variable with the largest w_+ is therefore likely to be the most important variable in the model (99) and best explains the variation in pathogen prevalence each scale across the range of *S. demersus*.

3. RESULTS

3.1 Verification of infection

Microscope analysis

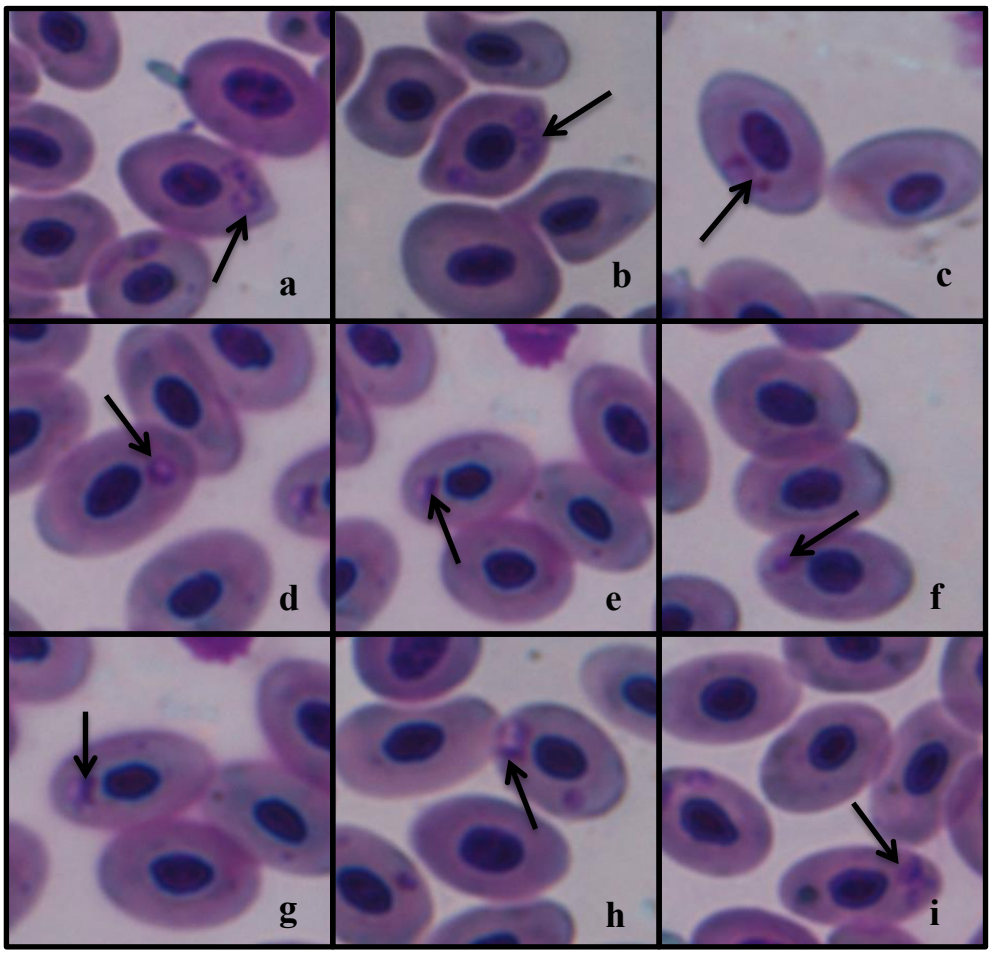

Figure 3 Photomicrographs of thin blood smears at 100× stained with Giemsa (1/10 *v*/v) after post-mortem examination. Several blood stages with consistent morphological characteristics of *Babesia* **A – C** and *Plasmodium* sp. **D – I** can be observed. Photographs **A – C** show various stages of the cyclical *Babesia* protozoa moult, developing from trophozoite rings, into the characteristic tetrad or 'Maltese-cross' morphology of merozoites. Photographs **D – E** show asexual blood stages consistent with trophozoites of *Plasmodium*. Photograph **F** shows a young schizont and photograph **G** a mature schizont. Photographs **H – I** show gametocytes. In photograph **I**, note the displacement of the RBC nucleus.

Microscopic screening of known positive samples for *Plasmodium* and *Babesia* infection confirmed by the target species and that these protozoa were present at various stages in their life cycles.

Plasmodium sequencing

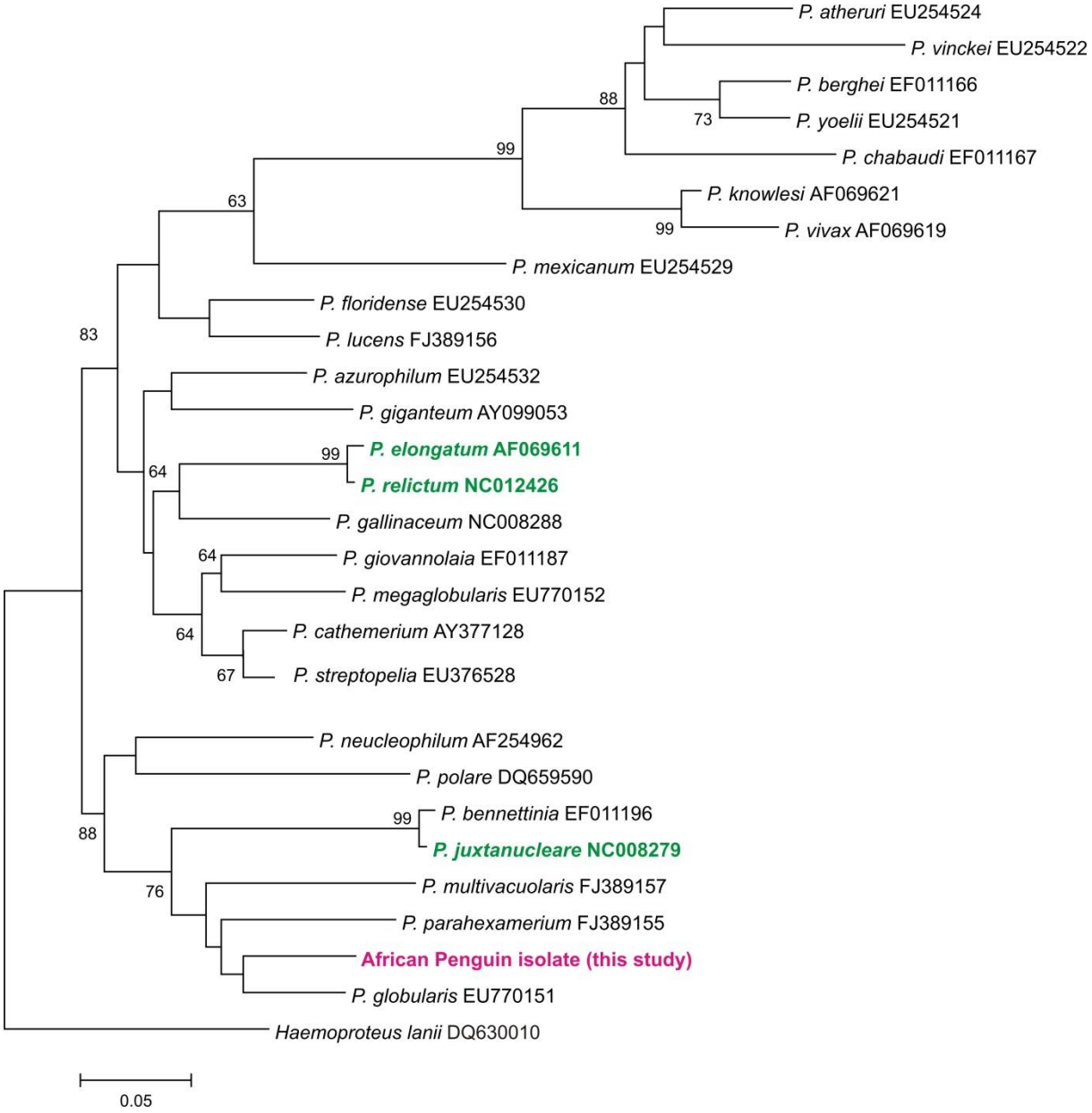

Figure 4 Phylogenetic placement of penguin positive controls for avian malaria infection using amino acid sequence data of the partial *cyt b* gene. *Haemoproteus lanii* (Genbank no. DQ630010) is used as the 'outgroup'. Bootstrap support values >50% are reported. Scientific names are given for parasite strains which have been morphologically described. The sequence isolate from this study is indicated on the figure. Species of *Plasmodium* that have been reported in wild and captive populations of African penguins are indicated in green. While *P. relictum* and *P. elongatum* occur naturally in breeding colonies, *P. juxtanucleare* has only been reported in captive penguins at SANCCOB; in all cases species identification was based on morphology only and not verified through DNA sequencing.

Sequence comparisons in both Genbank and MalAvi confirmed the penguin positive controls as most similar to lineages of avian *Plasmodium* species. Phylogenetic analysis of the control sequences

placed the penguin isolates clearly within the clade of avian *Plasmodium* species reported from terrestrial birds hosts [69].This confirms all controls as positive and all tests as negative for nested PCR primers used to diagnose both *Plasmodium* and *Haemoproteus* throughout the range.

3.3 Characterising *Babesia* prevalence

We determined the presence of *Babesia* infection for 317 individuals across the three breeding regions of African penguins. *Babesia* infection characterised all colonies analysed in the study (Figure 5), with 60% of individuals positive for infection.

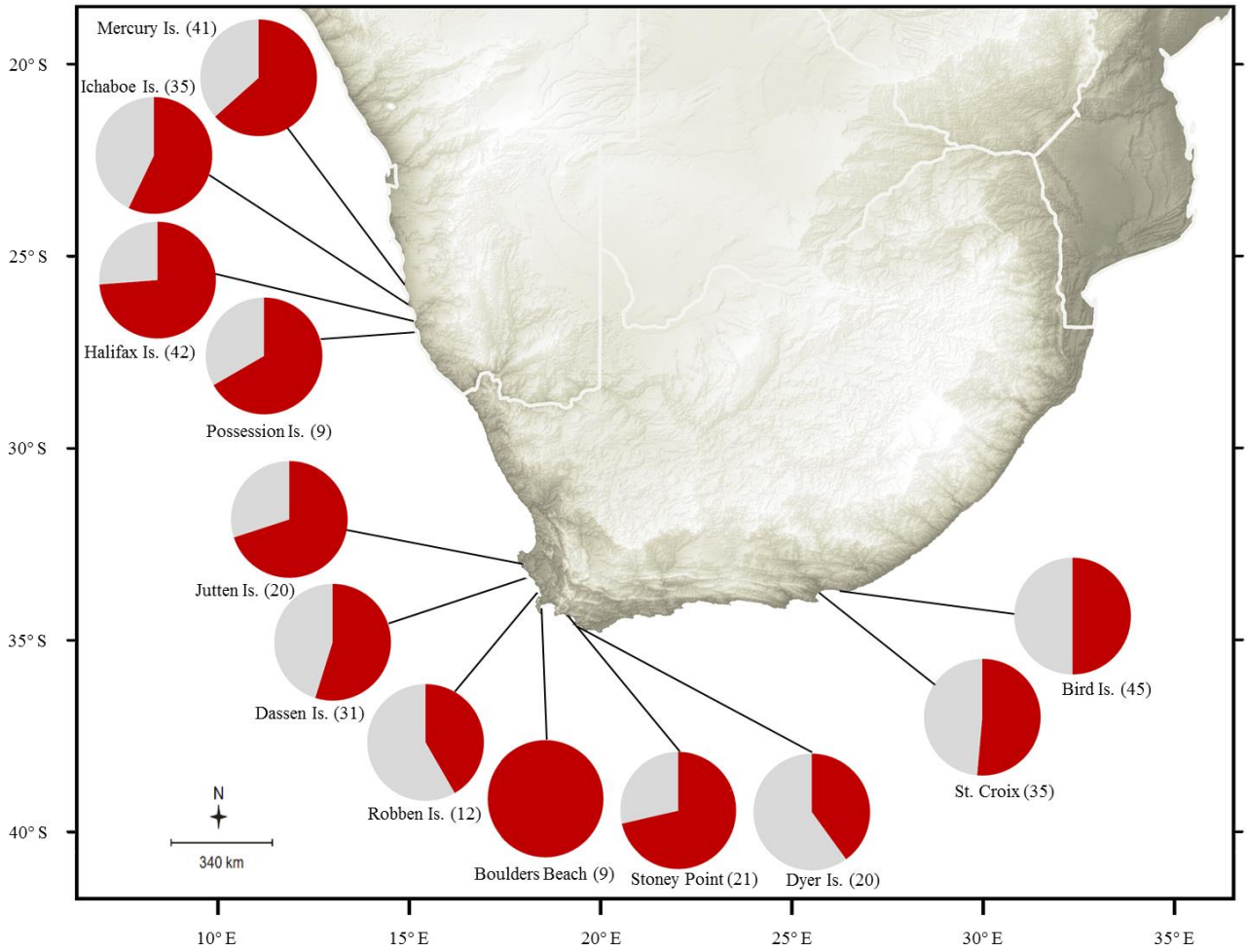

Figure 5 *Babesia* prevalence (%) diagnosed across all sampled island and mainland sites within the breeding regions of Namibia (Mercury, Ichaboe, Halifax and Possession Islands), the Western Cape (Jutten, Robben and Dyer Islands, also Boulders Beach and Stoney Point mainland sites) and the Eastern Cape (St. Croix and Bird Islands). The proportion of infected individuals per colony is depicted in red and sample sizes are represented with each colony label.

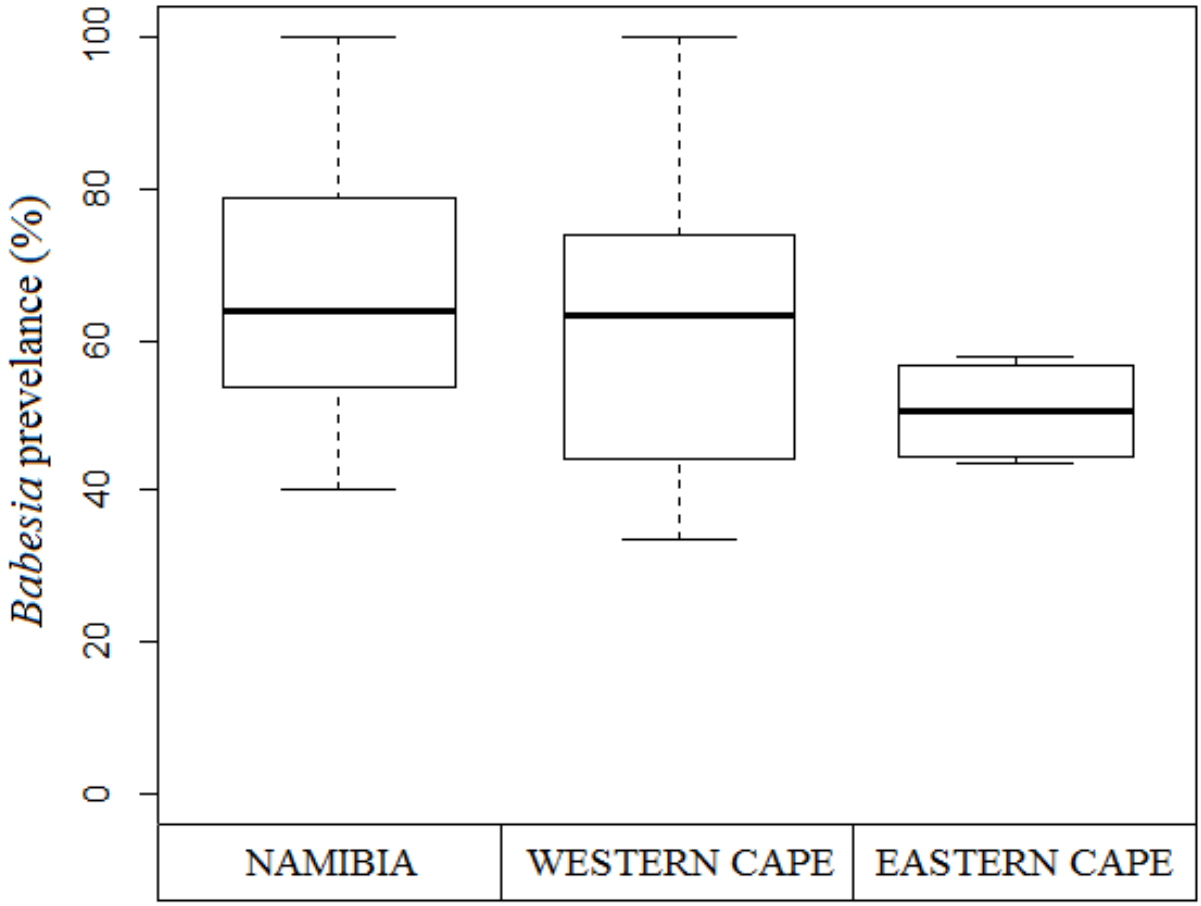

Figure 6 *Babesia* prevalence (%) diagnosed across all breeding regions of Namibia (Mercury, Ichaboe, Halifax and Possession Islands), the Western Cape (Jutten, Dassen, Robben and Dyer Islands, including the mainland sites of Boulders Beach and Stoney Point) and the Eastern Cape (St. Croix and Bird Islands).

The geographic distribution of infection revealed a general pattern of decreasing prevalence from Namibia (65%), through to 61% in the Western Cape to 51% in the Eastern Cape of South Africa.

3.4 Drivers of pathogen prevalence

Table 1 Akaike Information Criterion (AIC), AIC differences (Δ_i) and Akaike weights (w_i) of the three alternative geographic, colony and individual level-based models explaining the distribution in *Babesia* prevalence among 12 colonies of *Spheniscus demersus*.

Candidate models	AIC	Δ_i	Akaike weights (w_i)	ER	Scaled deviance
Geography model					
(1) LG + LS	**112.61**	**0**	**0.14**	-	23.78
(2) LT + LG + LS	113.35	0.74	0.10	1.45	44.51
(3) LT + LS	113.59	0.98	0.09	1.63	42.48
(4) LG	113.77	1.16	0.08	1.79	23.78
(5) LG + LS + SX	114.16	1.55	0.06	2.17	23.78
(6) LT + LG + LS + SX	115.03	2.42	0.04	3.35	42.48
(7) Reg (colony)	115.07	2.46	0.04	3.43	22.35
(8) Reg (colony) + LT	115.07	2.46	0.04	3.43	40.30
(9) Reg (colony) + LG	115.07	2.46	0.04	3.43	39.32
(10) LG + SX	115.41	2.80	0.03	4.06	22.35
…					
(25) LT + SX	117.60	5.00	0.01	12.15	21.69
Colony model					
(1) DM + LS	**104.62**	**0**	0.21	-	34.03
(2) DM	105.32	0.70	0.14	1.42	40.16
(3) DM + LS + SX	106.13	1.52	0.10	2.13	41.31
(4) DM + Ho + LS	106.38	1.76	0.09	2.41	34.01
(5) DM + CS + LS	106.61	1.99	0.08	2.70	34.03
(6) DM + SX	106.87	2.25	0.07	3.09	36.84
(7) DM + CS	107.30	2.68	0.05	3.82	31.33
(8) DM + Ho	107.32	2.70	0.05	3.86	39.36
(9) DM + Ho+ LS + SX	107.96	3.34	0.04	5.31	39.08
(10) DM + CS + LS + SX	108.12	3.50	0.04	5.74	31.32
…					
(25) Ho + SX	114.47	9.86	0.00	138.03	30.55
Individual model					
(1) SX	**118.09**	**0**	0.42	-	46.80
(2) LS	118.16	0.07	0.41	1.03	46.62
(3) LS +SX	119.93	1.83	0.17	2.50	46.63

LG, longitude (decimal degrees); LS, life stage (adult/chicks); LT, latitude (decimal degrees); SX, sex (male/female); Reg(colony), colony nested within the three breeding regions; DM, distance from mainland; Ho, observed heterozygosity; CS, colony size. The most parsimonious model at each level is highlighted in bold and those greyed models represent those with less empirical support.

The geography model

Babesia prevalence declines from west to east, whilst adults present with a higher level of *Babesia* infection than chicks overall along this trend.

Of the 25 alternative models considered as geographic factors influencing the distribution of *Babesia* prevalence, the model for longitude and its interaction with life stage has the lowest Δ_i and the highest w_i value. Based on the summed Akaike weights, longitude appeared to be the most important predictive variable in the geography model ($w_+ = 0.62$), as its interaction with life stage ($w_+ = 0.62$) has a 14% chance of being the best model and is 1.45x more likely than the next most parsimonious model including latitude as a factor (Table 1). Geography models two to five do however present considerable empirical support ($\Delta_i < 2$). Whereas the remaining models including the effects of individual colonies nested within each breeding region on prevalence have little empirical support ($\Delta_i > 2$).

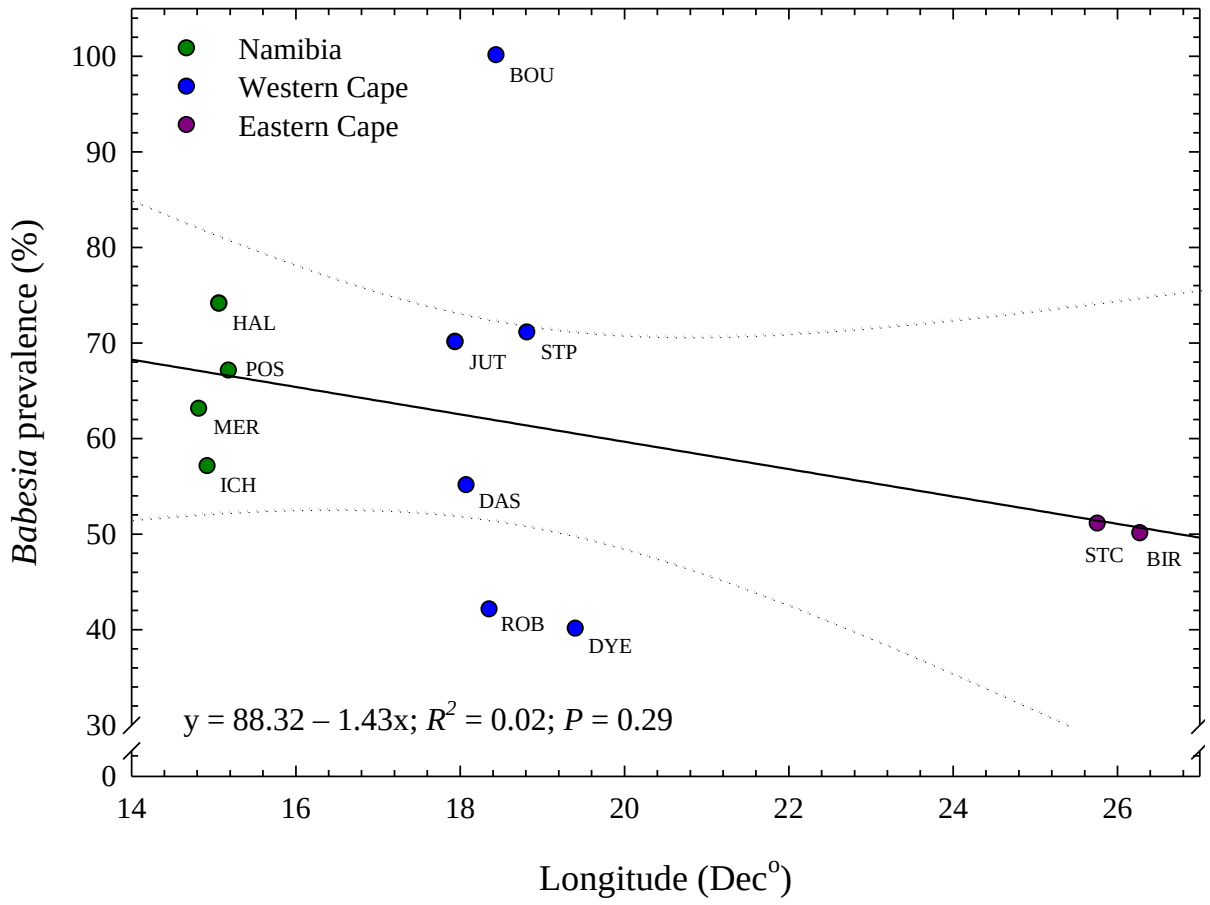

Figure 7 The relationship between the prevalence of *Babesia* (%) and longitude (decimal degrees). The regression of *Babesia* prevalence (%) and longitude (decimal degrees) is expressed. Depicted are the labels for each breeding colony within their respective breeding regions.

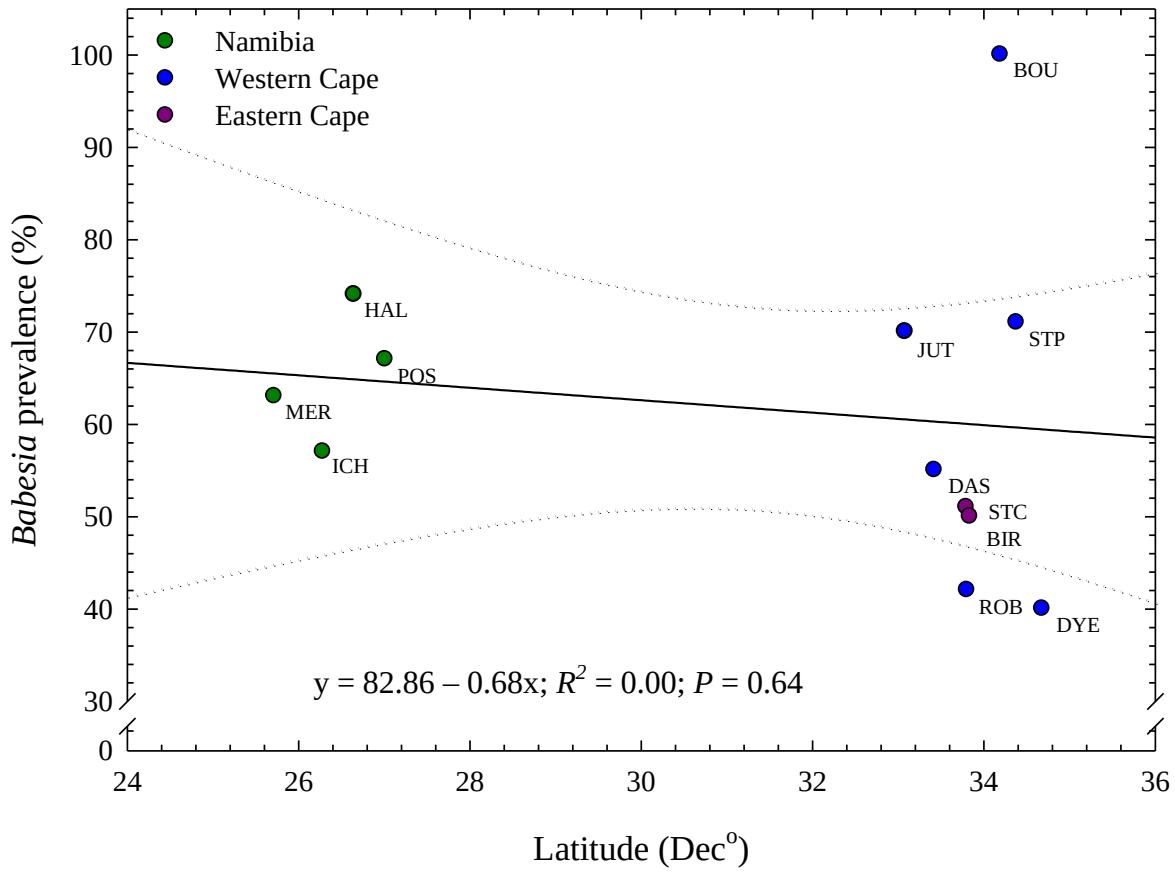

Figure 8 The relationship between the prevalence of *Babesia* (%) and latitude (decimal degrees). The regression of *Babesia* prevalence (%) and latitude (decimal degrees) is expressed. Depicted are the labels for each breeding colony within their respective breeding regions.

Summing the Akaike weights for the different covariates in these models shows that the longitude (w_+ = 0.62) and its interaction with life stage (w_+ = 0.62) is the most important predictive variable in the geography model, followed by latitude (w_+ = 0.47), individual colonies nested within each region (w_+ = 0.34) and finally sex (w_+ = 0.31).

Table 2 Wald's Chi-squared estimates and confidence intervals for the best-fit generalized linear model in the geographic model

Parameter	B Estimate (SE)	*df*	Wald χ^2	*P* –value	95% CI
Geography Model					
Longitude	0.085 (0.0313)	1	7.433	< 0.01	0.024, 0.146
Life stage	0.555 (0.3121)	1	3.158	0.076	-0.057, 1.166

The best-fit generalized linear model describes *Babesia* prevelance variation at a geographic level, as a function of longitude and life stage of *S. demersus*.

Babesia prevalence decreases with an increase in both longitude (Figure 7) and latitude (Figure 8), though these are not statistically significant linear associations ($P = 0.29$ and $P = 0.64$ respectively). The Wald's Chi-squared output (Table 2) suggests that of the parameters considered in the best fit generalized linear model of geography, only longitude ($P < 0.01$) has a significant association with *Babesia* prevelance (%). Life stage alone does not significantly influence prevalence ($P = 0.076$).

The colony model

Babesia prevalence declines exponentially with an increase in distance from the mainland (km), whilst adults present with a higher level of *Babesia* infection than chicks overall along this trend.

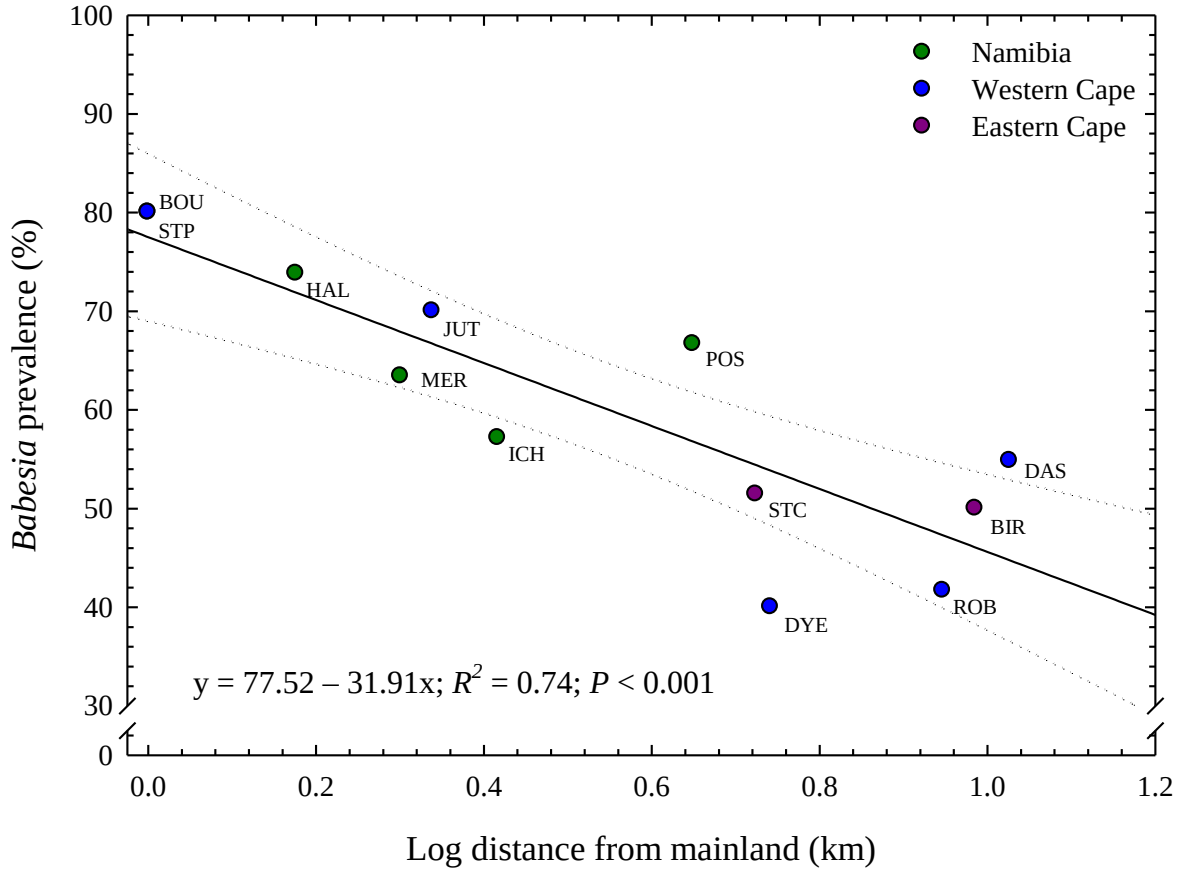

Figure 9 The relationship between the prevalence of *Babesia* and distance from the mainland. The regression of *Babesia* prevalence (%) and distance from the mainland (log km) is expressed. Depicted are the labels for each breeding colony within their respective breeding regions.

In the colony model, the distance from the mainland ($w_+ = 0.92$) and its interaction with life stage ($w_+ = 0.60$) is suggested to be the most important predictive variable, though colony models two to five do present with considerable empirical support ($\Delta_i < 2$). Models thereafter with a $\Delta_i > 2$ present as having little empirical support. The distribution of *Babesia* prevalence at a colony level seems to be primarily driven by each colony's distance from the mainland ($w_+ = 0.92$), as the model on which it is based has the lowest Δ_i and the highest w_i value of the 25 candidate models (Table 1). It has a 21% chance of being the best model and is 1.42x more likely than the next most parsimonious model. Summing the Akaike weights for the different covariates included in these models, we've shown that at a colony level, distance from the mainland is the most important predictor variable ($w_+ = 0.92$), followed by life stage ($w_+ = 0.60$), sex ($w_+ = 0.33$), the observed heterozygosity ($w_+ = 0.28$) and finally colony size ($w_+ = 0.27$).

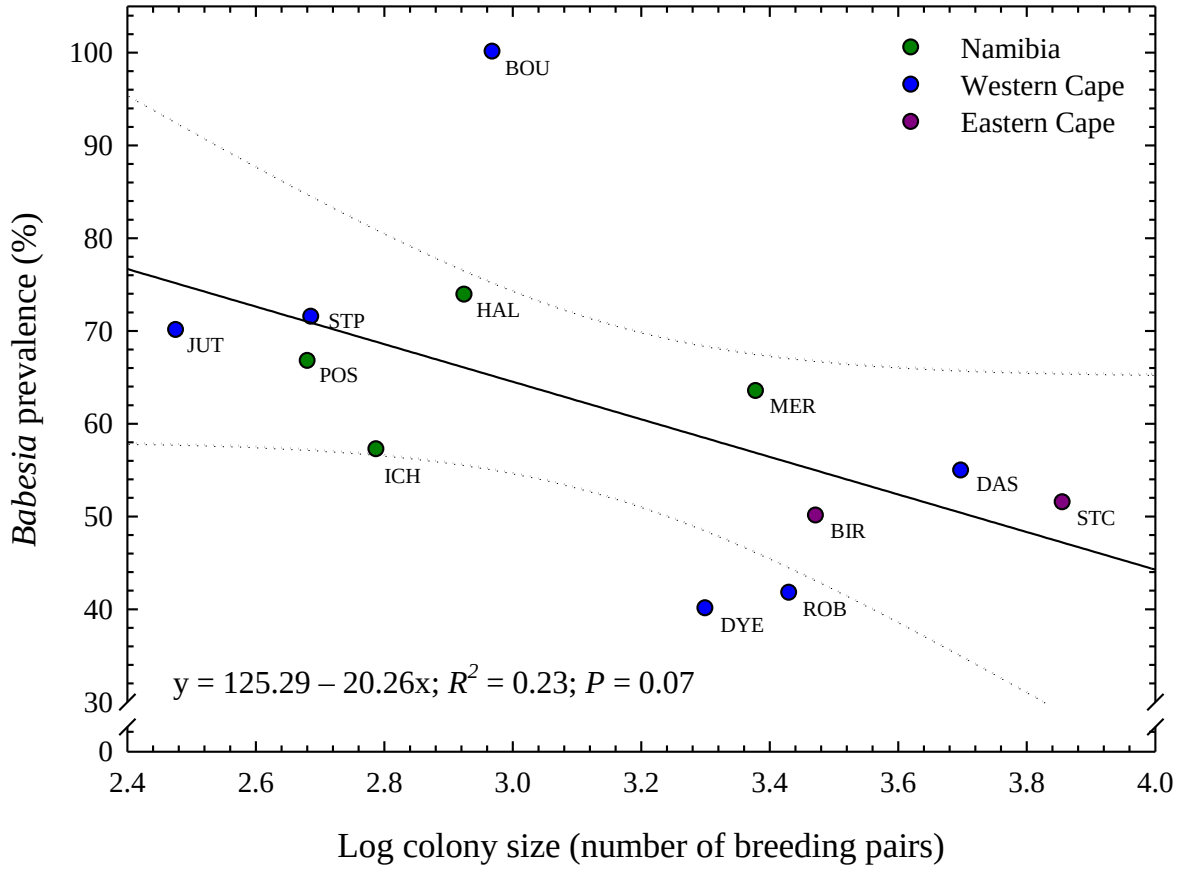

Figure 10 The relationship between the prevalence of *Babesia* and colony size. The regression of *Babesia* prevalence (%) and colony size (log number of breeding pairs) is expressed. Depicted are the labels for each breeding colony within their respective breeding regions.

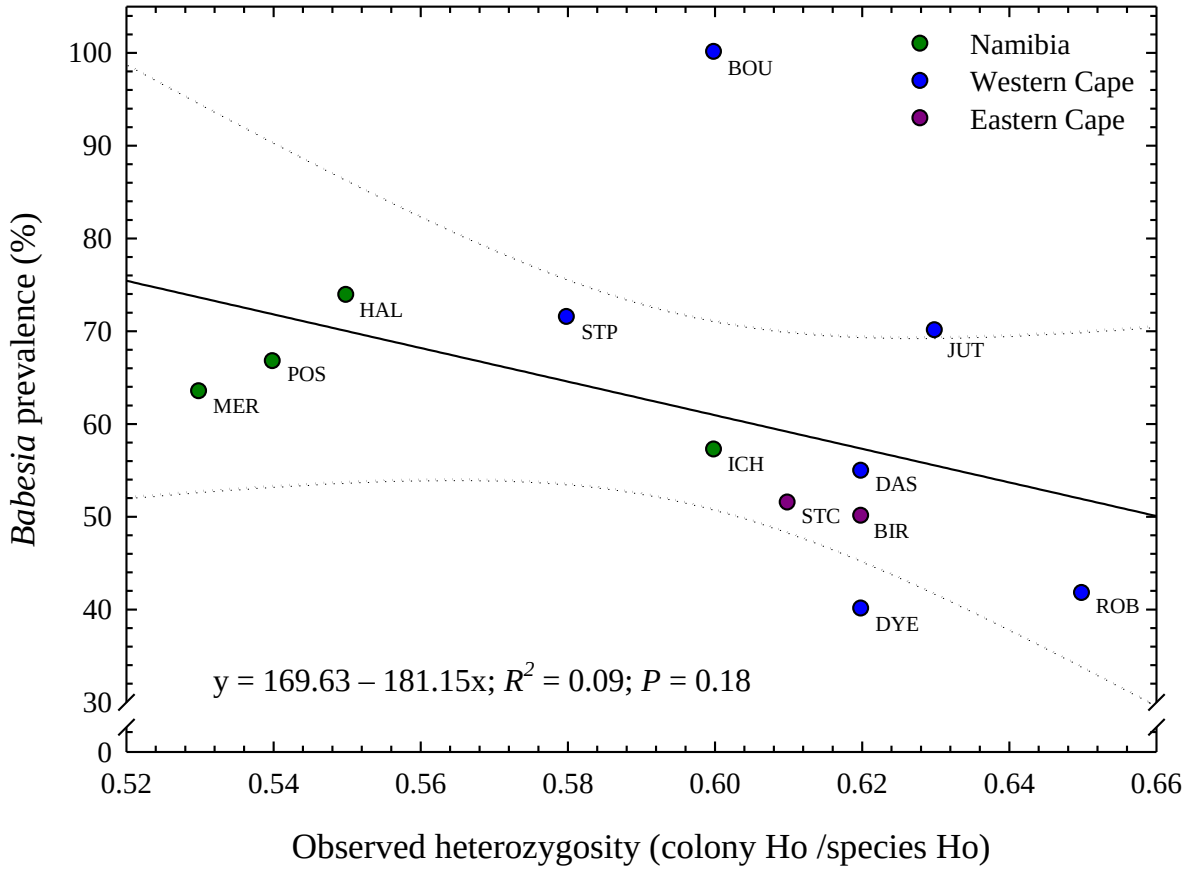

Figure 11 The relationship between the prevalence of *Babesia* and observed heterozygosity. The regression of *Babesia* prevalence (%) and heterozygosity (individual diversity/species diversity) is expressed. Depicted are the labels for each breeding colony within their respective breeding regions.

Table 3 Wald's Chi-squared estimates and confidence intervals for the best-fit generalized linear model in the colony model

Parameter	B Estimate (SE)	*df*	Wald χ^2	*P* –value	95% CI
Colony Model					
Distance from mainland	1.43 (0.38)	1	14.49	< 0.001	0.69, 2.16
Life stage	0.47 (0.29)	1	2.70	0.100	-0.09, 1.03

The best-fit generalized linear model describes *Babesia* prevelance variation at a colony level, as a function of distance from the mainland and life stage of *S. demersus*.

Babesia prevalence decreases with both log colony size (number of breeding pairs) and the observed heterozygosity for each island, though these trends do not depict significant linear relationships (P = 0.07 and P = 0.18) respectively. *Babesia* prevalence does however decrease significantly (P < 0.001) as the log distance from the mainland increases (Figure 11).

The Wald's Chi-squared output (Table 3) suggests that of the parameters considered in the best fit generalized linear model of geography, only the log distance from mainland ($P < 0.001$) has a significant association with *Babesia* prevelance (%), whereas life stage as an isolated factor has no significant effect on prevalence ($P = 0.1$).

The individual model

Neither the sex, life stage, nor the interactions between the two are significantly associated with *Babesia* prevalence.

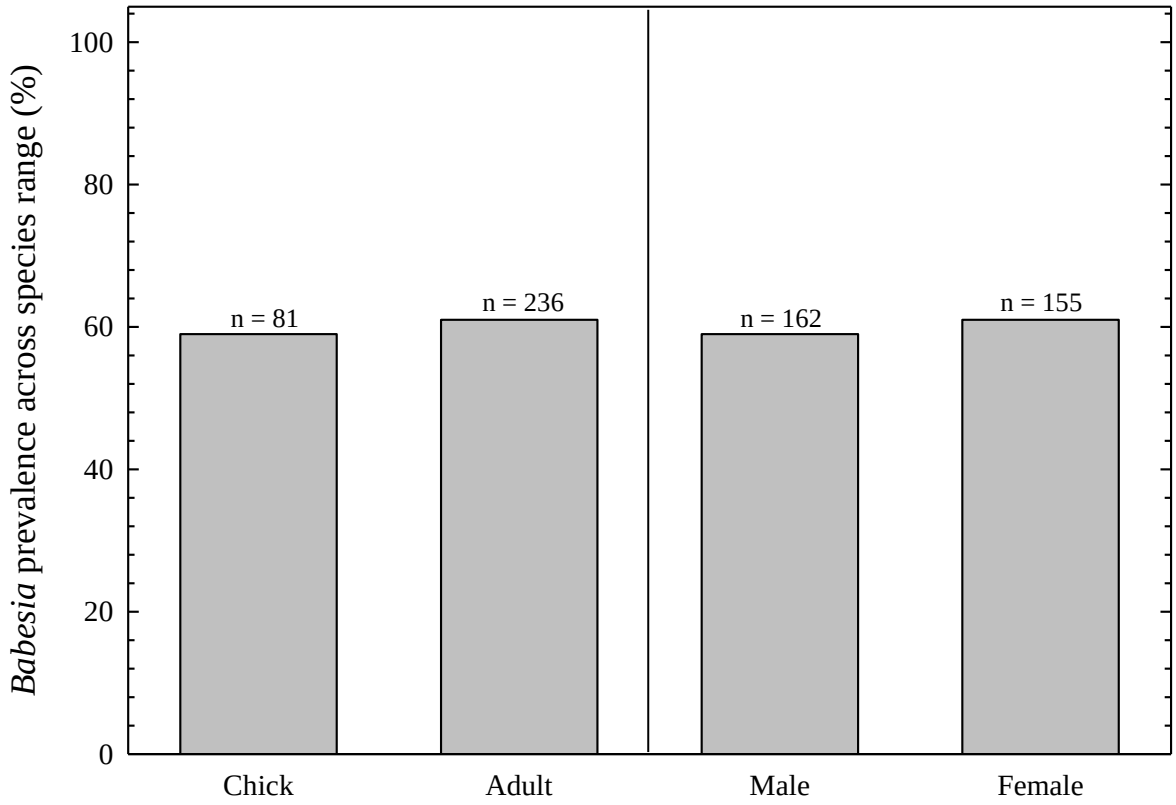

Figure 12 *Babesia* prevalence (%) diagnosed for all males and females across their breeding range. As the data are discrete, there is no error associated with its distribution per colony.

At the level of the individual, its sex ($w_+ = 0.59$) appears to be the most important factor influencing the distribution of *Babesia* prevalence, as this model has the lowest Δ_i and the highest w_i value of the three alternative models. The model of sex has a 42% chance of being the best model, but is only 1.03 times more likely than the model suggesting life stage (Table 1). The model based on the life stage of individuals, as well as its interaction with the sex of an individual does have considerable empirical support ($\Delta_i < 2$). Summing the Akaike weights for the different covariates in the individual

models shows that sex of the individual is the most important predictor variable (w_+ = 0.59), followed by its life stage (w_+ = 0.58), though this proximity means that they are equally likely to be primary drives.

Table 4 Wald's Chi-squared estimates and confidence intervals for the best-fit generalized linear model in the individual model

Parameter	B Estimate (SE)	*df*	Wald χ^2	*P* –value	95% CI
Model					
Sex	0.11 (0.23)	1	0.23	0.631	-0.34, 0.56
Life stage	0.107 (0.25)	1	0.17	0.682	-0.406, 0.620

The best-fit generalized linear model describes *Babesia* prevalence variation at an individual level, as a function of sex in *S. demersus*.

Babesia prevalence is higher in adults (61%) than in chicks (59%) and higher in females (61%) than in males (59%) throughout the breeding range of African penguins.

The Wald's Chi-squared output suggests that of the parameters considered in the best fit generalized linear model of individuals, neither the sex, life stage, nor the interaction between the two are significantly associated with *Babesia* prevalence.

4. DISCUSSION

African penguins are sentinels of marine health, not only as endemics to the southern African coastline, but to marine health globally. As major predators within the Agulhas-Benguela Ecosystem, they have a central role in the ecology of southern African waters and their decline is certainly a function of known and perhaps novel stresses on both the health and reproductive fitness of individual birds. Given that high levels of haemoparasitism routinely lead to increased morbidity and even mortality in captive birds [70], and the limited research into prevalence of haemoparasites in penguins, it is suggested that haemoparasitism may be of increasing concern in the conservation of African penguins, exacerbating the pressure on its remaining population. Using PCR-based screening of 317 birds, data presented here provides the first evidence for the extent of haemoparasite pathogen pressure experienced by African penguins across their geographic range. Furthermore, the analysis of potential ecological and abiotic parameters within a model framework provides novel insight into the possible drivers of pathogen prevalence in African penguins at different ecological scales.

4.1 Characterising pathogen prevalence

The data presented here reveal that infection with the tick-borne pathogen *Babesia* is generally high throughout the range of African penguins (60%, Figure 5). These findings corroborate those of Jones and Shellam (1999), which suggest that *Babesia* is, "endemic in *S. demersus* in southern Africa and is found regularly within the peripheral blood in local populations," though it should be noted that the samples analysed by these authors were taken only from captive birds [54]. Reports of such high incidence in wild birds are rare, even for the tropics (>30% [15]), as this atypical prevalence of *Babesia* deviates from a characteristically low prevalence in seabirds [16,32–35,71], as well as those from island [43] and marine [44–46] environments.

Unexpectedly, the PCR-based methods used in this study do not provide evidence for infection with *Haemoproteus* or *Plasmodium* species commonly borne by mosquitoes. The lack of *Haemoproteus* sp., despite being the most widespread and prevalent of blood protozoan in birds, serves to further substantiate the claim that it has not yet been recorded in wild or captive penguins [16,53]. In contrast the absence of avian malaria (*Plasmodium* sp.) throughout the range is anomalous and requires further investigation as to the sensitivity of the PCR-based method used. *Plasmodium* has commonly been reported in surveys of captive penguins of the genus *Spheniscus*, particularly from American zoos [72,73] and its prevalence in captive and rehabilitated individuals at SANCOBB is reported as ~ 15% [70,74]. Data presented here however support those of Brossy (1992) who

concluded that *Plasmodium* infection is rare in free-living African penguins in their natural habitat, suggesting an absence of suitable vectors and/or high post infection mortality within hosts.

4.2 Drivers of pathogen prevalence

Geographic scale

Babesia prevalence across the range of African penguins appeared to be best explained by the interaction between longitude and life stage, a model which was 3.43x more likely than the colony-specific effects predicted. The trend in prevalence was shown to decrease from west to east whilst adults showed a higher *Babesia* prevalence than chicks across this trend. There was also empirical support of latitudinal effects over those of colony specific influences, when interacting with life stage and longitude (1.45x). The *Babesia* prevalence was shown to decrease with latitude, supporting the findings of various authors. These suggest that this decline is probably due to the absence of suitable arthropod vectors at higher latitudes [16,48]. Such strong moderating effects on pathogen prevalence across a vast geographic range suggest that this trend may be linked to variations in climate, similar to the shifting 10°C isotherm theory proposed by Quillfeldt *et al*. [16,71], which may enable the range or density of arthropod vectors to be increased with a decrease in latitude. In addition to this, the parasites themselves may increase in number, as they reproduce more rapidly at higher temperatures [13]. Furthermore, food availability along the coastline of the Western Cape may have been reduced by El Niño Southern Oscillation events [9,75], and/or along the Namibian coast by an intense, unregulated commercial fishing policy in African penguin feeding grounds [76,77]. This may have increased stress on and reduced the physical condition in these birds, decreasing their resistance to infective challenges [13].

Colony scale

The prevalence of *Babesia* at the colony scale and indeed across all scales was best explained by a colony's distance from the mainland. This interacting with individual life stage produced a model which was 2.41x and 2.70x more likely than those of interactive models including the effects of colony heterozygosity and size respectively. *Babesia* prevalence showed a significant exponential decline as the distance from the mainland increased. Decreases in prevalence were also witnessed for increases in heterozygosity and surprisingly colony size.

The strong trend of decline as a colony's distance from the mainland increases is well supported in the literature, this is suspected to be the result of a marked reduction in the number of suitable vectors, as hindered by the sea barrier [13,71]. In addition, increasing disturbances at their mainland breeding sites by tourism, shipping, domestic and feral animals, as well as contact with a larger arthropod vector and reservoir population [78], engenders stress which may increase penguin susceptibility to disease if the pathogens are present. Furthermore increased exposure of penguins to environmental pollutants and effluent surrounding inhabited areas and industrial ports may compromise their immune systems and make them more susceptible to infective challenges [79].

Heterozygosity was measured through microsatellite markers. Though these are non-coding neutral markers, they have been used extensively as a general measure of genome variation. It follows that *Babesia* prevalence should increase with a reduction in this heterozygosity, as there is essentially a smaller pool of genetic diversity from which these African penguins can draw their immunogenetic resistance. Given the rapid and dramatic decline of this species, they have shown a surprisingly small reduction in genetic diversity and shown no signs of population bottlenecking effects, losing only ~10% of their diversity in the last decade [59]. This implies and corroborates findings supporting high levels of gene flow between colonies, despite strong patterns of breeding colony fidelity [12]. African penguins are thus thought to present life history traits which buffer the effects of genetic bottlenecking events which aid their persistence.

The decrease in *Babesia* prevalence with an increase in colony size is curious as it opposes current theory on density-dependent pathogen transmission [80]. Perhaps this is linked to vector saturation at a threshold of colony size, given the two distinct groupings of size (Figure 10). It is however recommended that this be a focal point of future research. The immediate implications for conservation management suggest that reintroduction into larger colonies may potentially offer improved shelter from haemoparasitic infection.

Individual scale

Whilst females appeared to present with a higher *Babesia* prevalence than males and adults more than chicks, there was no significant bias in either of these throughout the range of African penguins. Breeding is monogamous in this species and incubation is shared equally by both sexes [12]. Thus *Babesia* prevalence does not appear to be altered by the slight difference in size and foraging effort of females in the breeding season [58]. It was also expected that chicks would present with higher pathogen prevalence than adults, as they would not yet be able to preen themselves of ectoparasites

and would have long nestling periods were the potential exposure to arthropod vectors is high [16]. Instead, adults showed a non-significantly higher *Babesia* prevalence than chicks, suggesting that infection probability increases with cumulative exposure [49]. This is further supported by the significant increase of *Babesia* prevalence in birds closer to the shore. As those spending more time in the vicinity of the mainland are probably more exposed to vectors [16].

4.3 *Babesia, Ixodes* ticks and African penguins

Though the arthropod vector for *Babesia* has not been formally reported, Earlé *et al*. (1993) [54] suspected a species of tick (*Ixodes uriae*). The drivers of its prevalence presented in this study support this proposed arthropod as the primary vector for *Babesia* in African penguins. *Ixodes uriae* has a circumpolar distribution and has been shown to carry other haemoparasites to these colonies via polar birds [81]. A key feature to the potential success of ticks parasitising and infecting African penguins with the haemoparasites they carry, is penguin colonial breeding [82], which can lead to aggregations of thousands of birds during the breeding season [12]. These colonies are ideal for tick infestation as they provide spatially discrete habitats suited to shelter-seeking ticks and a source of food throughout the year. Moreover, the strong breeding site fidelity and relatively long chick rearing periods of African penguins provide a reliable and predictable host resource for ticks [57]. In addition, this tick species has the ability to moult (for larva or nymphs) or oviposit (females) and is thereby able to survive from year to year awaiting the host's return, building up large, resilient populations at these colonies over time [83]. It is suspected that the large populations of gannets, Cape- and Bank-cormorants, as well as other seabirds occurring in sympatry with African penguins serve as reservoirs of this *Babesia* infection.

4.3 Limitations, implications and recommendations

Limitations or Errors in Methods and Assumptions

Most previous studies made use of blood smears to detect haemoparasites; however it has been suggested that these may not detect parasites if the intensity is low (e.g. *Plasmodium* and *Haemoproteus* spp. during early spring; [84]). Instead, genetic methods using sensitive PCR-based screening can detect some parasitaemia missed by blood smears [61,65,85–88], however false negatives are not completely excluded by molecular methods [89]. It is suspected that the nested PCR-based method proposed by Hellgren *et al.* (2004)[62], used in this study is not specific enough to detect the range of *Plasmodium* sp. elsewhere detected within African penguins and thus [90,91],

in future, a genus specific primer should be used and confirmed with both sequencing and microscopy.

Boulders Beach – a call for research

The resident colony of African penguins at Boulders Beach is a flagship to the overall health and status of the species, generating up to R14.5 million in ecotourism per annum [92]. It is the accessibility and charisma of this colony that has placed it under a fortress conservation policy, preventing any sampling or research. The limited data presented for this colony however, undoubtedly sets apart from those throughout the range for all parameters considered. All individuals sampled were infected with *Babesia*, implying that the conditions at this colony are optimum for infection. It is therefore recommended that the conservation policy for this colony be reconsidered to permit research relating to haemoparasitism, as this will not only verify the arthropod vectors responsible for transmission, but will contribute to the understanding of haemoparasite interactions and effects thereof on African penguins as a species. This research will certainly serve to inform and improve the localised conservation management of this valuable colony.

Implications for captivity, conservation research and management

This baseline data on prevalence and relative ranking of its potential drivers at various scales across the breeding range of African penguins attempts to elucidate the role of pathogens and their vectors for this iconic host. Enhancing our understanding of these complex relationships will begin to improve not only the clinical treatment of captive or rehabilitated birds, but also inform the conservation of what is a small and rapidly declining wild population. Given the absence of *Plasmodium* species detected in this study, it is suggested that avian malaria in captive birds is primarily transmitted through vectors within and around the facility. It is believed that this transfer may be from other birds within the facility as opposed to having been contracted in the wild. If at all possible, it is recommended that rehabilitation programmes avoid freshwater sites where arthropod vector densities may be highest. In addition, given the high prevalence of *Babesia* infection throughout the range of African penguins, it is recommended that research be directed at quantifying the effects of chronic sublethal infections on the condition, reproductive fitness and relative survival rates of individual birds.

4.4 Conclusions

The nested method of PCR-based screening for *Plasmodium* and *Haemoproteus* species used in this study suggests that wild African penguins do not present with infection by these haemoparasites. *Babesia* species prevalence was however diagnosed as positive for > 60% of all birds sampled; a haemoparasitic incidence twice that of most tropical systems. The primary driver of this prevalence is shown to be the interaction of a colony's distance from mainland and the life stage of an individual. *Babesia* prevalence shows a significant exponential decrease with and increased distance from the mainland, where adults have a slightly greater *Babesia* prevalence then chicks across this trend. The literature suggests that this is primarily due to a scarcity of arthropod vectors along this trend. It is suggested that knowledge of these trends in haemoparasitic prevalence should be harnessed to revise policy regarding the protection of these endangered African penguins, such as at the Boulders Beach colony where *Babesia* prevalence was 100%. We have a responsibility and an obligation to ensure that changes to the environment of African penguins do not include the introduction of diseases to which these penguins are not at present exposed to or against which they demonstrate limited resistance.

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REFERENCES

1 Randall, R. M. (1989) Oceans of Life Off Southern Africa, ed. A.I.L. Payne and R.J.M. Crawford. In Oceans of Life Off Southern Africa, pp 244–256.

2 Shannon, L. J. and Crawford, R. J. M. (1999) Management of the African penguin *Spheniscus demersus*: Insights from modelling. Mar. Ornithol. **27**, 119–128.

3 Kemper, J., Underhill, L. G., Crawford, R. J. M. and Roux, J. P. (2007) Final Report of the BCLME (Benguela Current Large Marine Ecosystem) Project on Top Predators as Biological Indicators of Ecosystem Change in the BCLME, pp 325–342.

4 Malbrant, R. and Maclatchy, A. (1958) A propos de l'occurrence de deux oiseaux d'Afrique austral au Gabon: Le manchot du Cap, *Spheniscus demersus* Linné, et la grue couronnée, Balearica regulorum Bennett. L'Oiseau la Rev. Française d'Ornithologie **28**, 84 – 86.

5 Wilson, R., Wilson, M. and Duffy, D. (1988) Contemporary and historical patterns of African penguin *Spheniscus demersus*: Distribution at sea. Estuar. Coast. Shelf Sci. **26**, 447–458.

6 Rand, R. W. (1960) The biology of guano-producing seabirds: The distribution, abundance and feeding habits of the Cape penguin, *Spheniscus demersus*, off the south-western coast of the Cape Province. Investig. Rep. Sea Fish. Res. Inst. South Africa **41**, 1–28.

7 Shelton, P., Crawford, R. J. M., Cooper, J. and Brooke, R. K. (1984) Distribution, population size and conservation of the jackass penguin *Spheniscus demersus.* South African J. Mar. Sci., pp 217–257.

8 BirdLife International. (2010) Species factsheet: *Spheniscus demersus* [Online]. BirdLife International, Cambridge. http://www.birdlife.org. Acessed 29 September 2014.

9 Crawford, R., Altwegg, R., Barham, B., Barham, P., Durant, J., Dyer, B., Geldenhuys, D., Makhado, A., Pichegru, L., Ryan, P., et al. (2011) Collapse of South Africa's penguins in the early 21st century. African J. Mar. Sci. **33**, 139–156.

10 BirdLife International. (2013.) Species factsheet: *Spheniscus demersus* [Online]. BirdLife International, Cambridge. http://www.birdlife.org. Accessed 29 September 2014.

11 Department of Environmental Affairs. (2013). African Penguin Biodiversity Management Plan. Government Gazette. **36966**.

12 Crawford, R. J. M., Kemper, J. and Underhill, L. G. (2013) African Penguin (*Spheniscus demersus*). Zhurnal Eksp. i Teor. Fiz., pp 212 – 235.

13 Jones, H. I. and Shellam, G. R. (1999) Blood Parasites in penguins, and their potential impact on conservation. Mar. Ornithol. **27**, 181–184.

14 Bennett, G., Whiteway, M. and Woodworth-Lynas, C. (1982) A host-parasite catalogue of the avian haematozoa. Occas. Pap. Biol. Meml. Univ. Newfoundland, St. John's, Canada **5**.

15 Jones, I. (1985) Hematozoa from Montane Forest Birds in Papua New Guinea. J. Wildl. Dis. **21**, 7–10.

16 Quillfeldt, P., Arriero, E., Martínez, J., Masello, J. F. and Merino, S. (2011) Prevalence of blood parasites in seabirds - a review. Front. Zool., BioMed Central Ltd **8**, 26.

17 Bennett, G. F., Peirce, M. and Ashford, R. W. (1993) Avian Haematozoa: mortality and pathogenicity. J. Nat. Hist. **27**, 993–1001.

18 Sorci, G. and Moller, A. P. (1997) Comparative evidence for a positive correlation between haematozoan prevalence and mortality in waterfowl. J. Evol. Biol. **10**, 731–741.

19 Sol, D., Jovani, R. and Torres, J. (2003) Parasite mediated mortality and host immune response explain age-related differences in blood parasitism in birds. Oecologia **135**, 542–7.

20 Pape, A., Nielsen, J. T., Ecology, S., Apr, N., Marie, P. and Cedex, P. (2007) Malaria and Risk of Predation: A Comparative Study of Birds. Ecology **88**, 871–881.

21 Merino, S., Moreno, J., Sanz, J. J. and Arriero, E. (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). Proc. Biol. Sci. **267**, 2507–10.

22 Sanz, J., Arriero, E., Moreno, J. and Merino, S. (2001) Female hematozoan infection reduces hatching success but not fledging success in Pied Flycatchers (*Ficedula hypoleuca)*. Auk **118**, 750–755.

23 Sanz, J. J., Arriero, E., Moreno, J. and Merino, S. (2001) Interactions between hemoparasite status and female age in the primary reproductive output of pied flycatchers (*Ficedula hypoleuca)*. Oecologia **126**, 339–344.

24 Marzal, A., de Lope, F., Navarro, C. and Møller, A. P. (2005) Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia **142**, 541–5.

25 Knowles, S. (2010) Chronic malaria infections increase family inequalities and reduce parental fitness: experimental evidence from a wild bird population. J. Evol. Biol. **23**, 557–69.

26 Hõrak, P., Ots, I., Vellau, H., Spottiswoode, C. and Pape Møller, A. (2001) Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. Oecologia **126**, 166–173.

27 Del Cerro, S., Merino, S., Martínez-de la Puente, J., Lobato, E., Ruiz-de-Castañeda, R., Rivero-de Aguilar, J., Martínez, J., Morales, J., Tomás, G. and Moreno, J. (2010) Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*). Oecologia **162**, 825–35.

28 van Riper III, C., van Riper, S. G., Goff, M. L., and Laird, M. (1986) The epizootiology and ecological significance of malaria in Hawaiian land birds. Ecol. Monogr. **56**, 327–344.

29 Tella, J. L., Cortazar, C., Gajon, A. and Oscar, J. J. (1995) Apparent lack of effects of high louse-fly infestation (Diptera, Hippoboscidae) on adult colonial alpine swifts. Ardea **83**, 435–439.

30 Figuerola, J. A., Verlarde, A., Bertolero, A. and Cerda, F. (1996) Abwesenheit von Haematozoa bei einer Brutpopulation des Seeregenfeifers (*Charadrius alexandrinus*). J. fur Ornithol. **137**, 523–525.

31 Masello, J. F., Choconi, G., Seghal, R. N. M., Tell, L. and Quillfeldt, P. (2006) Blood and intestinal parasites in wild Psittaciformes: a case study of Burrowing Parrots (*Cyanliseus patagonus*). Ornitol. Neotrop. **17**, 515–529.

32 Pierce, M. A. and Brooke, M. (1993) Failure to detect blood parasites in seabirds from the Pitcairn Islands. Seabird **15**, 72 – 74.

33 Merino, S., Barbosa, A., Moreno, J. and Potti, J. (1997) Absence of haematozoa in a wild chinstrap penguin Pygoscelis Antarctica population. Polar Biol. **18**, 227 – 228.

34 Merino, S. and Minguez, E. (1998) Absence of haematozoa in a breeding colony of the storm petrel *Hydrobates pelagicus*. Ibis (Lond. 1859). **140**, 180 – 181.

35 Engström, H., Dufva, R. and Olsson, G. (2000) Absence of Haematozoa and ectoparasites in a highly sexually ornamentd species, the Crested Auklet. Waterbirds **23**, 486 – 488.

36 Bennett, G. F., Bishop, M. A. and Peirce, M. A. (1993) Checklist of the avian species of *Plasmodium* Marchiafa & Celli, 1885 (Apicomplexa) and their distributio by avian family and Wallacean life zones. Syst. Parasitol. **26**, 171 – 179.

37 Valkiu-nas, G., Salaman, P. and Iezhova, T. A. (2003) Paucity of Haemotozoa in Columbian birds. J. Wildl. Dis. **39**, 445 – 448.

38 Scheuerlein, A. and Ricklefs, R. E. (2004) Prevelance of blood parasites in European passeriform birds. Proc. R. Soc. London Ser. B **271**, 1363–1370.

39 Arriero, E. and Møller, A. P. (2008) Host ecology and life-history traits associated with blood parasite species richness in birds. J. Evol. Biol. **21**, 1504 – 1513.

40 Bennett, G. F., Montgomerie, R. and Seutin, G. (1992) Scarcity of haematozoa in birds breeding on the Arctic tundra of North America. Condor **94**, 289–292.

41 Little, R. M. and Earle, R. A. (1995) Sandgrouse (*Pterocleidae*) and Sociable Weavers (*Philetarius socius)* lack avian haematozoa in semi-arid regions of South Africa. J. Arid Environ. **30**, 367–370.

42 Valera, E., Carrillo, C., Barbosa, A. and Moreno, E. (2003) Low prevalance of haematozoa in Trumpeter finches *Bucanetes githangineus* from south-eastern Spain: additional support for a restricted distribution of blood parasites in arid lands. J. Arid Environ. **55**, 209–213.

43 Little, R. M. and Earle, R. A. (1994) Lack of avian haematozoa in the Phaisaninae of Robben Island. Ostrich **65**, 343–344.

44 Piersma, T. (1999) Do global patterns of habitat use and migration stratergies co-evolve with relative investment in immunocompetance due to spatial variation in parasite pressure? Oikos **80**, 623–631.

45 Figuerola, J. A. (1999) Effects of salinity on rates of infestation of waterbirds by haematozoa. Ecography (Cop.). **22**, 681–685.

46 Jovani, R., Tella, J. L., Forero, M. G., Bertelloti, M., Blanco, G., Ceballos, O. and Donazar, J. A. (2001) Apparent absence of blood parasites in the patagonian seabird community: Is it related to the marine environment? Waterbirds **24**, 430–433.

47 Barbosa, A. and Palacios, M. J. (2011) Health of Antarctic birds: a review of their parasites, pathogens and diseases. Polar Biol. **32**, 1095–1115.

48 Merino, S., Moreno, J., Vasques, R. A., Martinez, J., Sanchez-Monsalves, I., Estades, C. F., Ippi, S., Sabat, P., Rozzi, R. and McGhee, S. (2008) Haematozoa in forest birds from southern Chile: Latitudinal gradients in prevelance and parasite lineage richness. Austral Ecol. **33**, 329–340.

49 Mendes, L., Piersma, T., Lecoq, M., Spaans, B. and Ricklefs, R. E. (2005) Disease-limited distributions? Contrasts in the prevalence of avian malaria in shorebird species using marine and freshwater habitats. Oikos **109**, 396–404.

50 Martínez-Abain, A., Esparza, B. and Oro, D. (2004) Lack of blood-parasites in bird species: Does absence of blood parasite vectors explain it all? Ardeola **51**, 225–232.

51 Earle, R. A., Bennett, G. F. and Brossy, J. J. (1992) First African record of *Leucocytozoon tawaki* (Apicomplexa: Leucocytozooidae) from the Jackass Penguin *Spheniscus demersus*. South African J. Zool. **27**, 89 – 90.

52 Cunningham, M., Gibbs, P., Rogers, T., Spielman, T. and Walraven, E. (1993) Ecology and health of the Little Penguin Eudyptula minor near Sydney. A report prepared for the Sydney Water board, pp 1 – 12.

53 Jones, H. I. and Shellam, G. R. (1999) The occurance of blood-inhabiting protozoa in captive and free-living penguins. Polar Biol. **21**, 5–10.

54 Earle, R. A., Huchzermeyer, F. W., Bennett, G. F. and Brossy, J. J. (1993) *Babesia peircei* sp. nov. from the jackass penguin. South African J. Zool. **28**, 88–90.

55 Yabsley, M. J., Parsons, N. J., Horne, E. C., Shock, B. C. and Purdee, M. (2012) Novel relapsing fever Borrelia detected in African penguins (*Spheniscus demersus*) admitted to two rehbilitation centers in South Africa. Parasitol. Res **110**, 1125–1130.

56 Olsen, B., Duffy, D. C., Jaenson, T. G. T., Gylfe, A., Bonnedahl, J. and Bergstrom, S. (1995) Transhemispheric exchange of Lyme disease spirochetes by seabirds. J. Clin. Microbiol. **33**, 3270–3274.

57 Dietrich, M. (2011) Worldwide distribution and diversity of seabird ticks: implications for the ecology and epidemiology of tick-borne pathogens. Vector-borne and Zoonotic Diseases. **11**, 453–70.

58 Pichegru, L. and Parsons, N. (2014) Female-biased mortality in African penguins. African J. Mar. Sci. **36**, 279–282.

59 Nupen, L. (2014) A conservation genetic study of threatened, endemic southern African seabirds. (Unpublished)

60 Fridolfsson, A. K. and Ellengren, H. (1999) A simple and universal method for molecular sexing of non-ratite birds. J. Avian Biol. **30**, 116–121.

61 Quillfeldt, P., Martinez, J., Bugoni, L., Mancini, P. L. and Merino, S. (2014) Blood parasites in noodies and boobies from Brazilian offshore islands - differences between species and influence of nesting habitat. Parasitology **141**, 399–410.

62 Hellgren, O., Waldenstrom, J. and Bensch, S. (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium* and *Haemoproteus* from avian blood. J. Parasitol. **90**, 797–802.

63 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**, 4673–4680.

64 Hall, T. A. (1999) BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symp. Ser 95–98.

65 Bensch, S., Stjernman, M., Hasselquist, D., Ostman, O., Hannson, B. and Westerdahl, H. (2000) Host specificity in avian blood parasites: a study of *Plasmodium Haemoproteus* mitochondrial DNA amplified from birds. Proc. R. Soc. London Ser. B **267**, 1583–1589.

66 Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. **20**, 2725–2729.

67 Burnham, K. P. and Anderson, D. R. (2002) Model selection and multi-model inference: a practical information-theoretic approach. New York Springer-Verlag.

68 Symonds, M. and Moussalli, A. (2010) Model selection, multimodal inference and model averaging using Akaike's information criterion: an introduction for statistically terrified behavioural ecologists. Behav. Ecol. Sociobiol.

69 Valkiūnas, G., Iezhova, T. A., Loiseau, C., Chasar, A., Smith, T. B. and Sehgal, R. N. (2008) New species of haemosporidian parasites (Haemosporida) from African rainforest birds, with remarks on their classification. Parasitol. Res.

70 Grim, C. K., Van der Merwe, E., Sullivan, M., Parsons, N., McCutchan, T. F. and Cranfield, M. (2003) *Plasmodium juxtanucleare* Associated with Mortality in Black-Footed Penguins (*Spheniscus demersus*) Admitted to a Rehabilitation Centre. J. Zoo Wildl. Med. **34**, 250–255.

71 Quillfeldt, P., Martinez, J., Hennicke, J., Ludynia, K., Gladbach, A., Masello, J. F., Riou, S. and Merino, S. (2010) Hemosporidian blood parasites in seabirds - a comparative genetic study of species for Antarctic to tropical habitats. Naturwissenschaften **97**, 809–817.

72 Beier, J. C. and Stoskopf, M. K. (1980) The epidemiology of avian malaria in Black-footed Penguins. J. Zool. Anim. Med. **11**, 99–105.

73 Fix, A., Waterhouse, C., Greiner, E. C., Stoskopf, M. K. (1988) *Plasmodium relictum* as a cause of avian malaria in wild-caught Magellanic penguins (*Spheniscus magellanicus*). J. Wildl. Dis. **24**, 610–9.

74 Brossy, J.J. (1992) Malaria in Wild and Captive Jackass Penguins *Spheniscus Demersus* Along the Southern African Coast. Ostrich **63**, 10–12.

75 Scheiber, R. W. and Schreiber, E. A. (1984) Central Pacific seabirds and the El Nino Southern Oscillation. Science **225**, 713–715.

76 Woehler, E. J. (1995) Consumption of Southern Ocean marine resources by penguins. In The penguins: ecology and management (Dann, P., Norman, I., and Reilly, P., eds.), pp 266–295, Surrey Beatty & Sons, Chipping Norton.

77 Cordes, I., Crawford, R. J. M., Williams, A. J. and Dyer, B. M. (1999) Decrease of African penguins at the Possession Island group, 1956-1995: Contrasting trends for colonial and solitary breeders. Mar. Ornithol. **27**, 129–138.

78 Cooper, J., Avenant, N. L. and Lafite, P. W. (1994) Airdrops and King Penguins: a potential conservation problem at sub-Antarctic Marion Island. Polar Res. **30**, 277–282.

79 Luebke, R. W., Hodson, P. V., Faisal, M., Ross, P. S., Grasman, K. and Zelikoff, J. (1997) Aquatic pollution and induced immunotoxicity in wildlife species. Fundam. Appl. Toxicol. **37**, 1–15.

80 Begon, M., Hazel, S. M., Baxby, D., Bown, K., Cavanagh, R., Chantrey, J., Jones, T. and Bennett, M. (1999) Transmission dynamics of a zoonotic pathogen within and between wildlife host species. Proc. Biol. Sci. **266**, 1939–45.

81 Peirce, M. A. and Prince, P. A. (1980) *Hepatozoon albatrossi* sp. nov. (Eucoccida: Hepatozoidae) from *Diomedea* spp. in the Antarctic. J. Nat. Hist. **14**, 447–452.

82 Møller, A. P., Allander, K. and Dufva, R. (1990) Fitness effects of parasites on passerine birds: a review. In Population Biology of Passerine Birds: an Integrative Approach (Blondel, J., Goster, A., Lebreton, J., and McCleery, R., eds.), pp 269–280, Springer-Verlag, New York.

83 Danchin, E. (1992) The incidence of the tick parasite *Ixodes uriae* in kittiwake *Rissa tridactyla* colonies in relation to the age of the colony, and a mechanism of infecting new colonies. Ibis (Lond. 1859). **134**, 134–141.

84 Valkiūnas, G., Iezhova, T. A., Krizanauskiene, A., Palinauskas, V., Seghal, R. N. M. and Bensch, S. (2005) Avian malaria parasites and other haemosporidia, CRC press, Boca Raton.

85 Feldman, R. A. and Freed, L. A. (1995) A PCR test for avian malaria in Hawaiian birds. Mol. Ecol. **4**, 673.

86 Perkins, S. L. and Schall, J. J. (2002) A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. J. Parasitol. **88**, 978.

87 Ricklefs, R. E., Swanson, B. L., Fallon, S. M., Martinez-Abrain, A., Scheuerlein, A., Gray, J. and Latta, S. C. (2005) Community relationships of avian malaria in southern Missouri. Ecol. Monogr. **75**, 543–559.

88 Parker, P. G., Whiteman, N. K. and Miller, R. E. (2006) Conservation medicine on the Gala'pagos Islands: partnerships among behavioural population and vetrinary scientists. Auk **123**, 625–638.

89 Gomez-Diaz, E., Doherty, P. F. J., Duneau, D. and McCoy, K. D. (2010) Cryptic vector divergence masks vector specific patterns of infection: an example from the marine cycle of *Lyme borreliosis*. Evol. Appl. **3**, 391–401.

90 Thiart, H. (2005) Immunological and epidemiological investigations into avian malaria in the African penguin during rehabilitation and in breeding colonies, University of Stellenbosch.

91 Szöllsi, E., Hellgren, O. and Hasselquist, D. (2008) A cautionary note on the use of nested PCR for parasite screening - an example from avian blood parasites. J. Parasitol. **94**, 562–564.

92 Lewis, S. E. F., Turpie, J. K. and Ryan, P. G. (2012) Are African penguins worth saving? The ecotourism value of the Boulders Beach colony. African J. Mar. Sci. **34**, 497–504.

APPENDIX

Table A1 Sampling details across the three breeding regions.

Breeding Region	Location	Chicks Sampled		Adults Sampled		Total	Latitude	Longitude
		♀	♂	♀	♂			
Namibia	Mercury	22	14	1	4	41	25°43'13.47"S	14°49'58.02"E
	Ichaboe	14	19	-	2	35	26°17'22.21"S	14°56'11.69"E
	Halifax	3	4	19	16	42	26°39'3.50"S	15° 4'47.38"E
	Possession	-	-	5	4	9	27° 0'52.19"S	15°11'40.11"E
						127		
Western Cape	Jutten	1	-	8	11	20	33° 5'5.53"S	17°57'16.44"E
	Dassen	-	-	15	16	31	33°25'31.76"S	18° 5'16.91"E
	Robben	-	4	3	5	12	33°48'22.70"S	18°22'9.39"E
	Boulders	-	-	2	7	9	34°11'49.67"S	18°27'5.05"E
	Stoney Point	-	-	8	13	21	34°23'1.06"S	18°49'44.64"E
	Dyer	-	-	11	9	20	34°41'0.52"S	19°24'58.93"E
						113		
Eastern Cape	St. Croix	-	-	19	16	35	33°47'57.76"S	25°46'11.70"E
	Bird	-	-	24	18	42	33°50'30.04"S	26°17'13.87"E
						77		

Table A2 PCR conditions and primer pairs used in the molecular screening.

Target	Primers	Sequence 5' → 3'	Size bp	Annealing	Extension	Target gene region	Reference
Avian sex	2550F	GTTACTGATTCGTCTACGAGA	450	53°C / 45sec	72°C / 50sec	CHD1Z (♂) CHD1Z + CHD1W (♀)	Fridolfsson & Ellengren (1999) [60]
	2718R	ATTGAAATGATCCAGTGCTTG	600				
Babesia	Bab600F	TCGTAGTTGAACTTCTGCTG	797	58°C / 30sec	72°C / 60sec	18s rDNA	Quillfeldt *et al.* (2014) [61]
	IsospR	ATTGCCTCAAACTTCCTTGC					
Plasmodium & *Haemoproteus*	HAEMF	CATATATTAAGAGAAT-TATGGAG	580	50°C / 30sec	72°C / 45sec	*Cyt b*	Hellgren *et al.* (2004) [62]
	HAEMNR2	AGAGGTGTAGCATATC TATCT-AC					

Table A4 Data on breeding region, geographic location, site (I = Island, M = Mainland), distance of the site from the nearest mainland (km), average sea surface temperature at each site (°C), the colony size of each site (number of breeding pairs in 2009) and observed heterozygosity for each site (H_O). Number of individuals, age, sex (♂ = male, ♀ = female) and total individuals sampled at each site (N_{Total}) are also reported.

Breeding Region	Location	I/M	Dist. Mainland (km)	Avg. Sea Surface Temperature (°C)	Colony Size (No. breeding pairs)	Observed Heterozygosity	Adults Sampled			Chicks Sampled			N_{Total}
							♂	♀	Total	♂	♀	Total	
Namibia	Mercury	I	1.00	14.21	2398	0.53	4	1	5	14	22	36	41
	Ichaboe	I	1.61	15.56	615	0.60	2	0	2	19	14	33	35
	Halifax	I	0.50	15.82	843	0.55	16	19	35	4	3	7	42
	Possession	I	3.46	14.20	481	0.54	4	5	9	0	0	0	9
							26	25	51	37	39	76	127
Western Cape	Jutten	I	1.18	15.20	408	0.63	11	8	19	0	1	1	20
	Dassen	I	9.63	15.68	5138	0.62	16	15	31	0	0	0	31
	Robben	I	7.85	16.25	2415	0.65	5	3	8	4	0	4	12
	Boulders	M	0.00	15.89	704	0.60	7	2	9	0	0	0	9
	Stoney Point	M	0.00	17.70	487	0.58	13	8	21	0	0	0	21
	Dyer	I	4.52	15.99	1260	0.62	9	11	20	0	0	0	20
							61	47	108	4	1	5	113
Eastern Cape	St. Croix	I	4.30	20.15	6824	0.61	16	19	35	0	0	0	35
	Bird	I	8.67	18.55	2624	0.62	18	24	42	0	0	0	42
							34	43	77	0	0	0	77
Total Range							121	115	236	41	40	81	317

Table A5 Data on the number of infected adults and chicks per sex (♂ = male, ♀ = female), the infection frequency (%) in adults and chicks per sex, the overall infection frequency (%) for males and females, as well as per colony.

Breeding Region	Location	No. Infected Adults			No. Infected Chicks			N_{Total}	Adults Infected (%)			Chicks Infected (%)			Infection Frequency (%)		
		♂	♀	Total	♂	♀	Total		♂	♀	Total	♂	♀	Total	♂	♀	Total
Namibia	Mercury	2	0	2	10	14	24	26	50	0	40	71	64	67	67	61	63
(127)	Ichaboe	2	0	2	8	10	18	20	100	-	100	42	71	55	48	71	57
	Halifax	11	17	28	1	2	3	31	69	89	80	25	67	43	60	86	74
	Possession	4	2	6	0	0	0	6	100	40	67	-	-	-	100	40	67
		19	19	38	19	26	45	83	73	76	75	51	67	59	60	70	65
Western Cape	Jutten	8	5	13	0	1	1	14	73	63	68	-	100	100	73	67	70
(113)	Dassen	8	9	17	0	0	0	17	50	60	55	-	-	-	50	60	55
	Robben	3	1	4	1	0	1	5	60	33	50	25	-	25	44	33	42
	Boulders	7	2	9	0	0	0	9	100	100	100	-	-	-	100	100	100
	Stoney Point	9	6	15	0	0	0	15	69	75	71	-	-	-	69	75	71
	Dyer	4	4	8	0	0	0	8	44	36	40	-	-	-	44	36	40
		39	27	66	1	1	2	68	64	57	61	25	100	40	62	58	60
Eastern Cape	St. Croix	7	11	18	0	0	0	18	44	58	51	-	-	-	44	58	51
(77)	Bird	10	11	21	0	0	0	21	56	46	50	-	-	-	56	46	50
		17	22	39	0	0	0	39	50	51	51	-	-	-	50	51	51
Total Range		75	68	143	20	27	47	190	62	59	61	49	68	58	59	61	60

Figure A1 Molecular discrimination of sex in *Spheniscus demersus* specimens of known and unknown sex. The size standard (LD.) is the GeneRuler 100 bp DNA ladder (New England Biolabs® Inc., MA, UK). Positive controls are female (♀ at 450bp and 600bp) and male (♂ at 600bp) respectively. Females show two bands (CHD1W at 450 bp and CHD1Z at 600bp) separated by approximately 150 bp while males show only a single band. Negative control is labelled as NC.

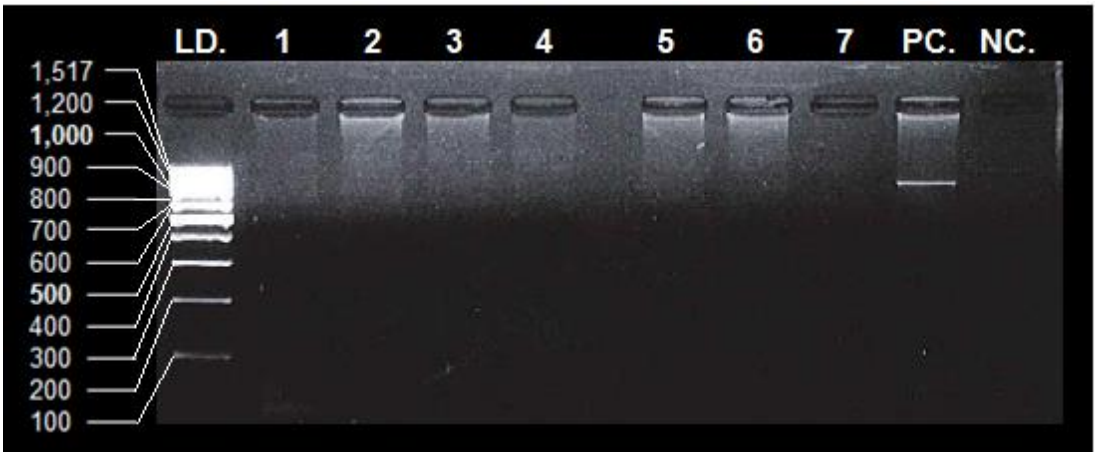

Figure A2 Molecular diagnosis of *Babesia* species in positive infection and unknown haemoparasitic status. The size standard (LD.) is the GeneRuler 100 bp DNA ladder (New England Biolabs® Inc., MA, UK). Positive control (PC.) for 18s rDNA in *Babesia* at approximately 830 bp. Individuals 1 through 7 are diagnosed as negative for all three haemoparasites. Negative control is labelled as NC.

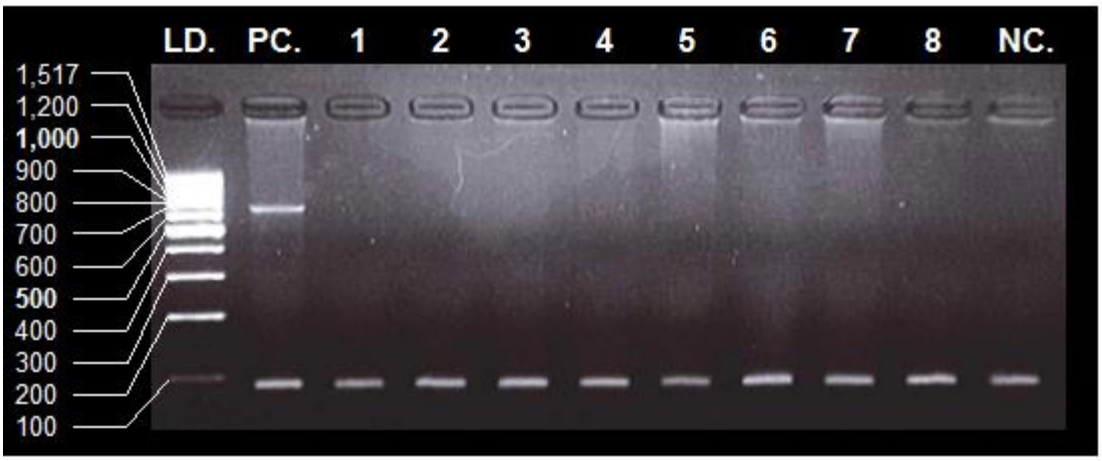

Figure A3 Nested molecular diagnosis of *Plasmodium*, *Haemoproteus* and *Leucocytozoon* species in specimens of positive infection and unknown haemoparasitic status. The size standard (LD.) is the GeneRuler 100 bp DNA ladder (New England Biolabs® Inc., MA, UK). Positive control (PC.) is *Plasmodium cyt b* specific amplification at approximately 600 bp. Individuals 1 through 8 are diagnosed as negative for all three haemoparasites. Negative control is labelled as NC.

Table A3 Correlation output for all covariate permutations.

Covariates	Correlation coefficient (r)	$T_{Statistic}$	*df*	*P* - value
Bab x Reg	-0.11	-2.05	315	0.041
Bab x Loc	-0.11	-1.97	315	0.050
Bab x Sex	0.03	0.48	315	0.632
Bab x LS	0.02	0.41	315	0.685
Bab x DM	-0.20	-3.65	315	< 0.005
Bab x CS	-0.15	-2.63	315	0.009
Bab x Ho	-0.13	-2.39	315	0.018
Reg x Loc	0.95	51.54	315	< 0.005
Reg x Sex	0.03	0.49	315	0.624
Reg x LS	0.58	12.68	315	< 0.005
Reg x DM	0.62	14.15	315	< 0.005
Reg x CS	0.56	11.89	315	< 0.005
Reg x Ho	0.70	17.31	315	< 0.005
Loc x Sex	0.02	0.38	315	0.702
Loc x LS	0.65	15.08	315	< 0.005
Loc x DM	0.55	11.57	315	< 0.005
Loc x CS	0.45	8.92	315	< 0.005
Loc x Ho	0.65	15.31	315	< 0.005
Sex x LS	-0.01	-0.10	315	0.919
Sex x DM	0.05	0.90	315	0.367
Sex x CS	0.07	1.32	315	0.187
Sex x Ho	-0.06	-1.02	315	0.310
LS x DM	0.29	5.42	315	< 0.005
LS x CS	0.17	3.07	315	0.002
LS x Ho	0.40	7.72	315	< 0.005
DM x CS	0.69	17.13	315	< 0.005
DM x Ho	0.65	15.10	315	< 0.005
CS x Ho	0.23	4.17	315	< 0.005