Host associations, biogeography, and phylogenetics of avian malaria in southern African waterfowl

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SUMMARY

The relevance of spatial variation in the environment and host communities for parasite community composition is poorly documented, creating a need for additional case studies from which general principles can be developed. Avian malaria in southern African waterfowl has not previously been studied. As a first step towards documenting and understanding its biogeography, we used PCR and molecular sequencing techniques to analyse 454 blood samples from Afrotropical ducks from 5 different locations (spread around the subregion) for avian malaria. Fifty-five blood samples were positive for one or more genera of haematozoa. The regional infection rate across all sites and sampling periods was 12·1%. Nine individuals carried dual infections containing multiple haematozoa. Fifteen different cytochrome *b* haplotypes among 52 positives (3 samples failed to sequence) and 61 total sequences were found. Eleven haplotypes closely matched *Plasmodium*, whereas 4 were more similar to *Haemoproteus*. Five distinct haematozoan clades were identified. *Haemoproteus* parasites appeared to be more host-specific than *Plasmodium*, which occurred at every sampling location and in every host species examined. There were no significant differences in overall parasite prevalence attributable to either site or species, although *Plasmodium* and *Haemoproteus* occurrences differed by site-species combination and the borderline significance of our test for between-site variation ($P<0·06$) implied that with a larger sample size, differences in parasite prevalence among locations might be detectable.

Key words: malaria, waterfowl, southern Africa, parasite, pathogen, evolution, biogeography, ecology.

INTRODUCTION

Analysis of the causes and consequences of spatial variation in the composition of ecological communities has been an important theme in the development of ecological theory. We can be confident for most vertebrates, for example, that biodiversity at a given location is a consequence of processes at multiple temporal and spatial scales (Gaston, 2000), and that dispersal and movement play critical roles in structuring animal communities (Hanski, 1998). Similarly, the relative importance of species sorting processes within a given area, such as competition, predation or disease, is contingent on resource availability, ecosystem productivity (Loreau *et al*. 2001), the magnitudes of both spatial and temporal variation (Rahbek, 2005), and the degree to which the ecosystem is open or closed (Ewers and Didham, 2006).

For some important kinds of community, however, general principles relating to spatial variation are less evident. One such puzzle piece is that of the parasite community (Lambin *et al*. 2010). There are at least 3 important questions relating to spatial variation in parasite communities to which we do not yet have definitive general answers. First, to what extent is spatial variation in most parasite communities driven by the external environment versus the composition of the vector and/or host communities? Second, how do feedbacks from parasites to their hosts and vectors influence spatial patterns of host biodiversity? And third, given that parasites often co-evolve with their hosts and vectors (Fallon *et al*. 2005), and may evolve more rapidly than either, how do the (usually) long-term dynamics of evolution interact in both space and time with the shorter-term ecological dynamics of host movements and community change?

Although a few research programmes have addressed some or all of these questions for particular systems (Loiseau *et al*. 2012), the field as a whole lacks well-established general principles that explain spatial variation in parasite communities. One of the critical needs in this context is thus for focused case studies that will help researchers to understand the distinctions between true general principles and case-specific detail. For example, the concept of dilution (i.e., the idea that if host competence differs, the

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transmission potential of parasites will be lower in a more diverse animal community) has been well supported for Lyme disease (e.g., Keesing *et al*. 2006), but its more general validity is not supported in many other host-parasite systems (Randolph and Dobson, 2012).

In this paper we present a first analysis of a previously little-explored case study system, that of waterfowl and avian malaria in southern Africa. Waterfowl offer a potentially interesting test case because they spend much of their time in wetlands that are highly suitable for mosquitoes; they are known to move widely throughout the subregion (Hockey, 2000); and most of the Afrotropical waterfowl species move only within the African continent and therefore remain throughout the year in areas in which avian malaria is endemic. The host community exhibits high levels of both spatial and temporal variation and the system thus provides valuable opportunities for examining the relevance of environmental variation and host movement patterns for host-parasite interactions, landscape epidemiology, and parasite biogeography.

All 3 genera of avian malaria haematozoa – *Plasmodium, Haemoproteus* and *Leucocytozoon* – occur in the subregion (Valkiūnas, 2005). *Plasmodium* spp. and *Haemoproteus* spp. were previously believed to have no detrimental effects on the avian host's health, but recent data suggest that infections by these genera can reduce survival, health status, and reproductive performance in the infected host (Marzal *et al*. 2008).

Given the nearly complete lack of background knowledge concerning our study system, we focus in this first analysis on 4 relatively straightforward questions: (1) how common is avian malaria in waterfowl within the southern African region, and does its prevalence vary significantly across the region? (2) Is avian malaria predominantly host specific, location specific, or some combination of the two? (3) Are there seasonal differences in the occurrence of avian malaria, based on expected changes in populations of insect vectors? And (4), are there consistent differences between sites or species in occurrences of the major genera of avian malaria (*Haemoproteus* and *Plasmodium*)? To answer these questions, we examined haematozoa infections in 454 individual birds from 8 waterfowl species (family Anatidae) belonging to 4 genera (*Anas*, *Alopochen*, *Plectropterus*, and *Dendrocygna*) that were sampled at 5 different locations in southern Africa.

MATERIALS AND METHODS

Field sites, study species, and sampling protocols

Blood samples were collected from 454 individual birds from 5 locations (Fig. 1): Strandfontein Wastewater Treatment Works (STR), near Cape Town in the Western Cape Province of South Africa;

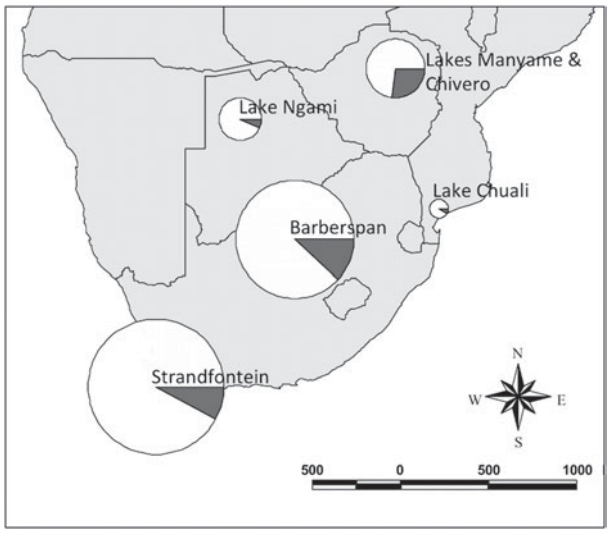

Fig. 1. Locations in southern Africa from which blood samples were collected. In each case, the centre of the pie chart matches the location of the site. Pie size is proportional to the number of samples that were tested; red shading indicates the number of samples that were positive for any haematozoa and white shading indicates negatives. Actual numbers are given in Table 1. Distances on the Scale bar are in kilometres.

Barberspan (BAR), near Delareyville in the Northwest Province of South Africa; Lake Manyame and Lake Chivero (ZW), adjacent man-made dams near Harare, Zimbabwe; Lake Chuali (CHU), about 100 km north of Maputo in Mozambique; and Lake Ngami (NGA), the southern end of the Okavango system in Botswana.

The 8 waterfowl (Anatidae) species included in this study were Cape Shoveler (*Anas smithii*), Cape Teal (*Anas capensis*), Hottentot Teal (*Anas hottentota*), Red-billed Teal (A*nas erythrorhyncha*), Yellow-billed Duck (*Anas undulata*), Egyptian Goose (*Alopochen aegyptiaca*), Spur-winged Goose (*Plectropterus gambensis*), and White-faced Whistling Duck (*Dendrocygna viduata*). Birds were captured using walk-in traps, mist nets, and cannon nets. Blood samples were collected by pricking the tarsal vein and capturing a small amount of blood in a capillary tube. Blood was transferred from the tube immediately into lysis buffer and stored in a sealed vial. Sampling was undertaken regularly over the course of 2 full calendar years, with 3 sites (Strandfontein, Barberspan, and Zimbabwe) being sampled every 2 months and 2 sites (Lakes Chuali and Ngami) being sampled every 4 months. Full details of the timing and location of each sampling event have been published by Cumming *et al*. (2011).

Molecular analysis

DNA extraction was carried out in the laboratory using the DNeasy tissue kit protocol (Quiagen).

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Following the protocol of Waldenström *et al*. (2002), a nested polymerase chain reaction was used to target a 478 bp fragment of the mitochondrial cytochrome *b* gene from the genera *Haemoproteus* and *Plasmodium*; the primers used do not amplify DNA from *Leucocytozoon* spp. (while we would have liked to include phylogenetic analysis of *Leucocytozoon*, costs were prohibitive). The reaction mix consisted of 8 μl of water, 12·5 μl of GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA), 1·5 μl of primer (10 nM) Haem NF (5′- CATATATTAAGAGAATTATGGAG-3′), 1·5 μl of primer (10 nM) Haem NR2 (5′-AGAGGTGTAGCATATCTATCTAC-3′), and 2 μl of DNA extract for a total reaction volume of 25 μl. The PCR profile was as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 sec, 50 °C annealing for 30 sec, and 72 °C extension for 45 sec, and finally a 72 °C step for 10 min. A second, nested PCR was then performed with 1·5 μl of the first PCR product and the nested set of primers (10 nM) Haem F (5′- AATGGTGCTTTCGATATATGCATG-3′) and Haem R2 (5′-GCATTATCTGGATGTGATAATGGT-3′), using the same reaction mix and PCR profile as above. We used 1·5% gel electrophoresis (agarose gels) to test for the presence of a PCR product indicative of a positive infection. Positive PCR products were cleaned with 1·8 μl of Agencourt Ampure beads following the manufacturer's protocols (Beckman Coulter, Danvers, MA, USA). PCR products were sequenced using Big Dye v.3.1 dye-terminator chemistry following the manufacturer's protocols (Applied Biosystems, Valencia, CA, USA). Automated sequencing was performed at the DNA sequencing facility on Science Hill at Yale University on an ABI 3730. Two trials were conducted for each sample. Sequences were aligned and edited using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA).

A Bayesian phylogeny of haematazoa haplotypes was constructed using MrBayes vers. 3.2.1 (Ronquist *et al*. 2012) and a general time reversible (GTR) substitution model with invariant sites (pinvar) and a gamma distribution of among-site rate variation (alpha). For this analysis, we ran 2 000 000 generations, sampling parameters and trees every 1000 generations; we discarded the first 25% of samples (i.e., 500 000 generations) as burn-in. The analysis was replicated which produced similar results and split frequencies were less than 0·01 at the end of each run. We also examined the partitioning of molecular variation among species and among sites using analyses of molecular variance (AMOVA; Excoffier *et al*. 1992) in the program Arlequin 3.1 (Excoffier *et al*. 2005). Species and sites were tested in separate AMOVAs. For each analysis, we excluded categories (species and sites, respectively) that contained fewer than 4 infections.

To examine evidence for the specificity of haematozoan lineages to Anseriformes, available cytochrome b sequence data for southern African birds were downloaded from GenBank. After excluding all accessions that had less than 300 bp of data, we obtained a total of 67 sequences from South Africa ($N=18$), Botswana ($N=42$), and Zimbabwe ($N=7$). Sequences were obtained from Waldenström *et al*. (2002), Ishtiaq *et al*. (2006, 2012), Beadell *et al*. (2006), and Durrant *et al*. (2007). Most sequences were isolated from avian hosts from the order Passeriformes (perching birds; $N=62$), but also from Coraciiformes ($N=2$), Columbiformes ($N=1$), Pelecaniformes ($N=1$), and Sphenisciformes ($N=1$), and sequences included both *Haemoproteus* spp. ($N=38$) and *Plasmodium* spp. ($N=29$). Including the 61 sequences obtained from Anseriformes for a total of 128 sequences, we constructed a Bayesian phylogeny using MrBayes vers. 3.2.1 as described above, except that we ran a total of 3 000 000 generations.

Statistical analysis

Impressions of prevalence and host specificity can be strongly influenced by sampling biases. Despite virtually constant sampling effort, our samples did not include a representative or standardized sample of the birds present at each site, for the simple reason that some species in some locations were extremely difficult to catch. Similarly, although we sampled year-round over a 2-year period, abundances of birds varied considerably and for most species there was a single time of year (often corresponding to the period before, during, or after flightless moult) when individuals were most abundant and easiest to catch at our study sites. To further complicate the analysis, the processes driving duck abundance are not synchronized across the region, with substantial variation occurring in the timing of rainfall and periods of high and low water. Notable differences in hydrology occur not only between summer and winter rainfall sites but also as a consequence of the 'delayed' flow regime of Lake Ngami (which receives most of its water from the Angolan highlands, via the lower end of the Okavango Delta). Although we started with simple calculations of overall prevalence across all species, more rigorous analysis and interpretation of the data had to be undertaken to correct for biases deriving from differences in location, species composition, and sampling time. All analyses were run in Matlab R2010b (Mathworks, 2010).

The methods for each of our focal questions are now considered in turn. The first was how common is avian malaria in waterfowl within the southern African region and whether its prevalence varies significantly across the region. To address this question we quantified both overall regional prevalence (i.e., pooling all samples into a simple overall statistic) and prevalence by site-species combination (i.e., prevalence within each species at each site). We tested for

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significant variation in prevalence among our study sites using an ANOVA by site of the site-species prevalence data, ignoring seasonal differences.

Our second question was that of whether there were seasonal differences in the occurrence of avian malaria in waterfowl. To test for a seasonal effect while correcting for sampling biases, we used a Mann-Whitney U-test (given that a Lilliefors test of the site-species-month prevalence data indicated significant departure from normality, $P<0{\cdot}001$) to compare the prevalence for each 'actual' species-site-month combination against a null hypothesis that assumed that the number of positives would be a consistent proportion of the total number of birds of a given species sampled at that site in each month. This analysis used only data for bird species that were sampled in a given month at a given site. Because of sample size constraints, we ran the analysis using data from all duck species and including all positives (i.e., data from both *Plasmodium* spp. and *Haemoproteus* spp.). The 28 rows of data for which both observed and expected values were 0 were removed from this analysis to avoid over-estimation of degrees of freedom (and hence, biasing the results towards significance), leaving a sample size of 58 observations for this test. We did not have a sufficiently large sample size to search for seasonal trends in data from individual sites.

Our third question was that of whether avian malaria is predominantly host specific, location specific, or some significant combination of the two. The challenge in addressing this question is again to correct for sampling bias. It can be re-phrased as the question of whether variation in malaria prevalence can be mainly ascribed to species influences, site influences, or a combination factor of the two. Given the (insignificant) outcome of the analysis for seasonal differences in malaria prevalence, we were justified in ignoring seasonality and comparing species and site effects using an N-way ANOVA (implemented through the Matlab procedure *anovan*) with prevalence as the response measurement and site and species as factors. We used the N-way ANOVA in preference to the two-way ANOVA because it is more robust to missing data, which were common in our data set because of differences in bird captures between sites (unsampled species cannot simply be assigned a prevalence of zero).

Our fourth question was that of whether there are consistent differences between sites or species in occurrences of the 2 major genera of avian malaria (*Haemoproteus* and *Plasmodium*). We tested for differences using data for all birds that tested positive for either parasite, first running a Mann-Whitney U-test to determine whether parasite occurrences for all unique site-species combinations differed and then an N-way ANOVA to test whether either site or species, as factors, explained a significant proportion of variance in the numbers of either parasite.

RESULTS

In the first PCR trial, using 454 individual birds, positive infections of haematozoa were detected in 46 (10·1%) individuals (Table 1). In the second trial, positives were detected in 34 (7·5%) individuals. Twenty-six individuals were positive in both trials, whereas 29 positives were either negative or ambiguous in one of the trials. Overall, 55 samples were positives in at least one trial. Nine individuals that were positive for haematozoa showed polymorphisms in their sequences. These samples were sequenced again with new PCR product to test for the possibility of contamination in the first trial. Upon verification that there were multiple peaks in the sequences, they were labelled as dual infections, containing multiple haematozoa. Two sequences were then designated for each of the individuals by comparing nucleotide polymorphisms between the ambiguous sequence and all resolved haplotypes. In each case, we were able to identify 2 haplotypes that, when combined, resulted in the same polymorphic sites as observed in the ambiguous sequence.

Fifteen different cytochrome *b* haplotypes were found among 52 positives (3 samples failed to sequence) and 61 total sequences (Fig. 2). GenBank BLAST searches revealed that 11 haplotypes closely matched *Plasmodium* spp., whereas 4 haplotypes were more similar to *Haemoproteus*. Of the 9 individuals carrying dual infections, 5 were infected by 2 lineages of *Plasmodium*, 3 were infected by 2 lineages of *Haemoproteus*, and 1 was infected by both *Plasmodium* and *Haemoproteus*. Based on posterior support of $\geqslant 0{\cdot}95$, five distinct clades (A–E) can be identified (Fig. 2), although 3 individuals with unique haplotypes did not fit into these clades. Sixteen *Haemoproteus* spp. parasites were detected and sequenced, revealing 4 different haplotypes. Ignoring sampling bias for the moment, *Haemoproteus* spp. infections appeared to be most common in Egyptian Geese ($n=11$; 68·8% of *Haemoproteus* spp. infections), but were also found in Cape Teal ($n=2$; 12·5%), Yellow-Billed Duck ($n=2$; 12·5%), and Red-Billed Teal ($n=1$; 6·3%). Fifteen of the 16 *Haemoproteus* spp. parasites were isolated from sampling localities in South Africa (BAR and STR) and the remaining one from the site in Bostwana (Figs. 2 and 3). No *Haemoproteus* spp. were detected in the Zimbabwean and Mozambican sites, the 2 most eastern sites. With 45 sequences, *Plasmodium* spp. showed a wide range of infection hosts and locations, occurring at every sampling location and in every host species examined. Although lineages C and D were most prevalent in Yellow-billed Ducks, lineages A and B were both found in a wide variety of hosts. Spur-winged geese from Zimbabwe showed a particularly high infection by *Plasmodium* spp., with infections being detected in 6 of the 7 individuals sampled (Fig. 3), 3 of which

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Table 1. Results, by site and species, giving the number of birds sampled of each species and the number that were positive for the genera *Plasmodium* and *Haemoproteus*

(Blank entries indicate zero prevalence rather than missing data. For clarity, the dual and unidentified infections were excluded from this table. Site names are STR, Strandfontein; BAR, Barberspan; MAN, Lake Manyame; CHI, Lake Chivero (MAN and CHI were merged into ZIM for the analysis); NGA, Lake Ngami; CHU, Lake Chuali.)

Site and Species	Birds Sampled	Positive for *Plasmodium*	Positive for *Haemoproteus*
BAR	148	12	4
Cape Shoveler	17		
Cape Teal	18		
Egyptian Goose	35	1	4
Red-billed Teal	22	4	
White-faced Duck	6		
Yellow-billed Duck	50	7	
CHI	20	6	
Egyptian Goose	5	1	
Spur-winged Goose	7	3	
White-faced Duck	8	2	
CHU	23	2	
Hottentot Teal	4		
Red-billed Teal	5		
White-faced Duck	10	2	
Yellow-billed Duck	4		
MAN	41	7	
Cape Shoveler	1	1	
Egyptian Goose	6		
Hottentot Teal	10	2	
Red-billed Teal	22	3	
White-faced Duck	2	1	
NGA	44	2	1
Egyptian Goose	4		
Hottentot Teal	13		
Red-billed Teal	21	2	1
White-faced Duck	6		
STR	178	5	5
Cape Shoveler	6		
Cape Teal	17	2	3
Egyptian Goose	103	1	2
Red-billed Teal	25	1	
Spur-winged Goose	10		
Yellow-billed Duck	17	1	
Total	454	34	10

contained multiple lineages (Fig. 2). Interestingly, all positive infections from the north-eastern sites (Zimbabwe, $N=21$ infections; Lake Chuali, $N=2$ infections) were *Plasmodium* lineages A and B, compared to 23·6% (9 of 38 infections) elsewhere in southern Africa.

The regional infection rate across all sites and sampling periods was 12·1%. When considered without corrections for sampling biases, regional infection rates varied significantly among host species ($\chi^2=19{\cdot}6$, D.F. $=7$, $P<0{\cdot}01$) and sampling locations ($\chi^2=17{\cdot}62$, D.F. $=4$, $P<0{\cdot}001$) (Fig. 3). In addition, 37% of the total molecular variation was partitioned among the 6 species that had $\geqslant 4$ infections (AMOVA, $P<0{\cdot}00001$), and 38% was partitioned among the 3 sites that had $\geqslant 4$ infections ($P<0{\cdot}00001$). Similar values were obtained when confining analyses to *Plasmodium* lineages only (32% among 5 species and 36% among 3 sites; $P<0{\cdot}00001$ in both cases).

The mean avian malaria prevalence value calculated for all site-species combinations was 16%. This value was heavily influenced by the fact that the single sampled Cape Shoveler from Zimbabwe was positive, giving 100% prevalence for this site-species combination. With Cape Shoveler in Zimbabwe removed, site-species mean prevalence was 12·6%. Haematozoan prevalence across all unique site-species-month combinations did not differ significantly from a null data set that assumed an even distribution across species within a site by month (Mann-Whitney U-test, $n=58$, $P<0{\cdot}14$), suggesting that there was no regional (overall) seasonal trend in malaria infections, although this test does not correct for regional differences in rainfall.

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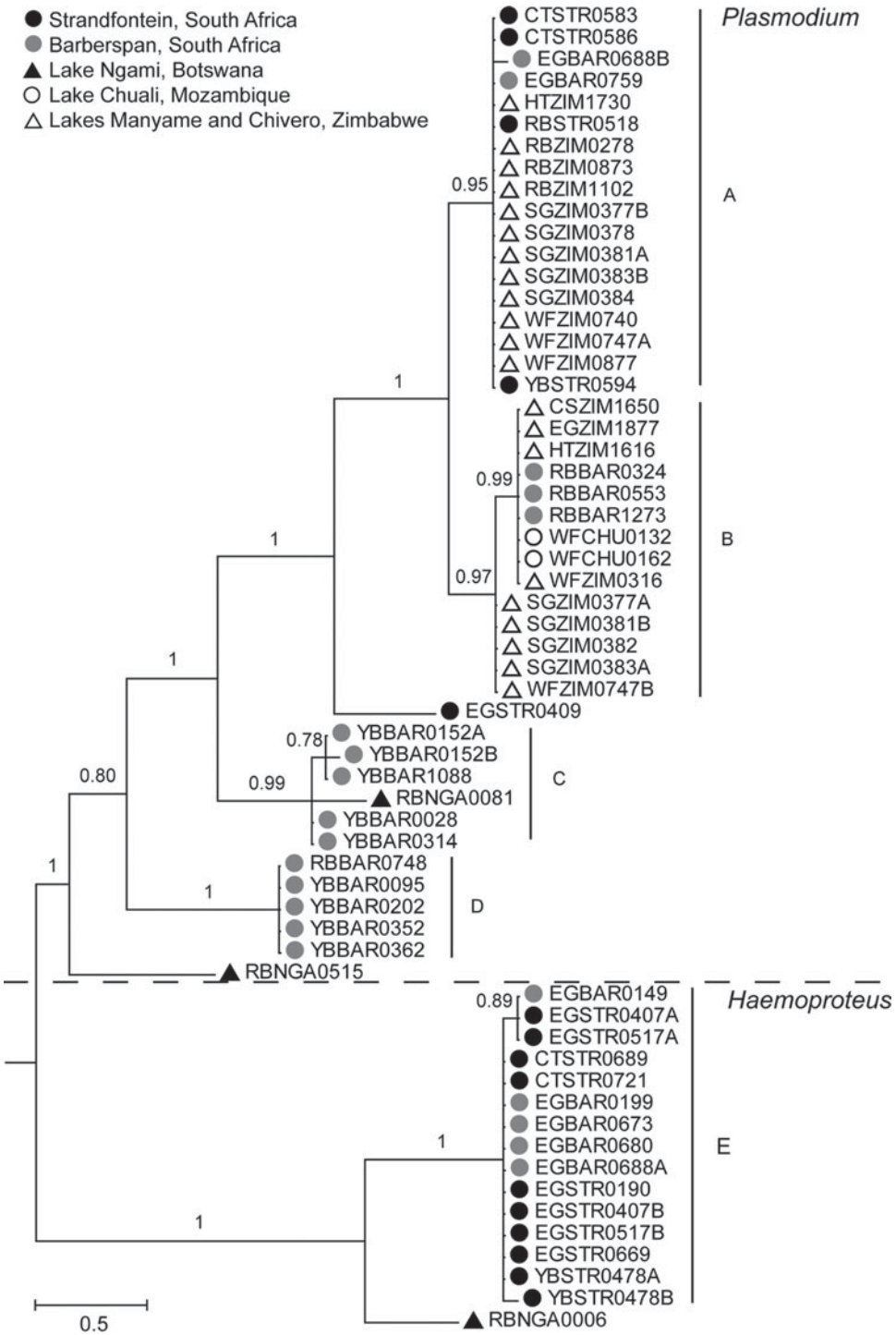

Fig. 2. Bayesian phylogenetic tree of mtDNA cytochrome *b* haplotypes obtained from haematozoa sample from waterfowl (pinvar = 0·46, alpha = 0·091). Sample names ending in an A or B indicate individuals that were infected by multiple haplotypes. Letters next to each vertical line indicate specific mtDNA lineages supported by >95% bootstrap support and that are referenced in the text. The horizontal dashed line separates *Plasmodium* and *Haemoproteus* haplotypes. The first 2 letters in each label indicate host species; Cape Shoveler (CS), Cape Teal (CT), Red-billed Teal (RT), Hottentot Teal (HT), Egyptian Goose (EG), Spur-winged Goose (SW), and White-faced Whistling Duck (WF). Numbers above branches indicate posterior nodal support.

An N-way ANOVA indicated that there were no significant differences in prevalence attributable to either site or species ($n = 26$; $F = 0{\cdot}79$, $P < 0{\cdot}6$ for species; $F = 2{\cdot}92$, $P < 0{\cdot}06$ for site). Unfortunately our data set was not large enough to quantify interaction effects between factors. The greater F value for site as a factor, and its borderline significance, suggest that a larger sample size might support the argument that differences in parasite levels between locations are greater than the differences between host species.

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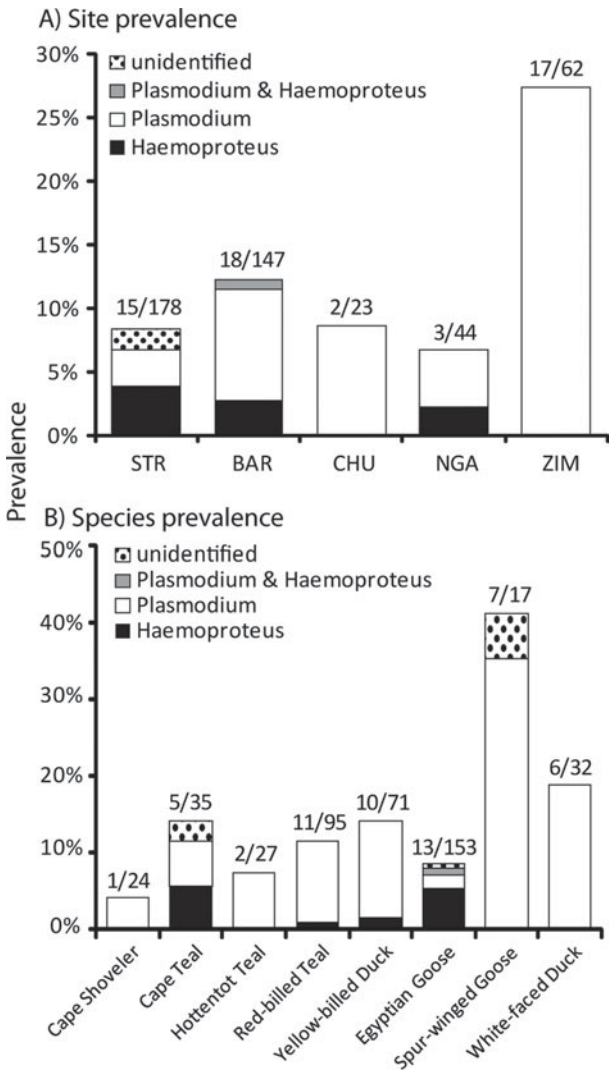

Fig. 3. Haematozoa infections by (A) location and (B) host species. The numbers above each column indicate the number of positive samples and the number that were tested.

Analysis of differences between infections for the two different haematozoa genera, *Plasmodium* and *Haemoproteus*, indicated significant differences in parasite occurrences by site-species pairs (Mann-Whitney U-test, $P<0{\cdot}0015$, $n=16$). However, tests of each individual data set for differences by site and species did not indicate a significant explanatory effect of either factor (N-way ANOVA, $n=16$; for *Plasmodium* spp., for species and site respectively, $F=0{\cdot}86$ and $0{\cdot}79$; $P<0{\cdot}6$ in both cases; for *Haemoproteus* spp., for species and site respectively, $F=2{\cdot}08$ and $1{\cdot}42$; $P<0{\cdot}29$ and $0{\cdot}41$).

Comparing haematozoan lineages isolated from Anseriformes with those isolated from other birds from southern Africa, we identified 2 major groups (1 *Haemoproteus* spp. and 1 *Plasmodium* spp.) and 1 minor group (*Plasmodium* spp.) that were exclusive to Anseriformes (Fig. 4). These monophyletic groups collectively included 4 of the lineages ('A', 'B', 'D' and 'E') identified in Fig. 2, as well as 2 of the 3 unique haplotypes that did not fit into any lineage. Lineage 'C', however, was present in both Anseriformes and Passeriformes and included a haplotype that was shared between the two orders. The *Plasmodium* sp. isolate from RBNGA0515 (Red-billed Teal, *Anas erythrorhyncha*) did not group with other Anseriformes lineages, but rather grouped with isolates from Passeriformes, Pelecaniformes, and Coraciiformes with high posterior probability ($0{\cdot}99$; Fig. 4). With the exception of lineage 'C', all Anseriformes isolates were well differentiated from haematozoa from other orders. Furthermore, 1 isolate from Pelecaniformes had an identical sequence to isolates from several Passeriformes, whereas haematozoa obtained from Coraciiformes, Columbiformes, and Sphenisciformes were unique and well differentiated from other isolates.

DISCUSSION

Given that avian malaria prevalence in southern African waterfowl has not previously been documented, our results show some interesting patterns. Avian malaria occurs in waterfowl at an average prevalence of around 12% across southern Africa. Infection with *Plasmodium* spp. appears to be approximately 3 times more common than infection with *Haemoproteus* spp. Infections in waterfowl in our study seem to have a non-random distribution with respect to the mtDNA lineage of haematozoa. Interestingly, waterfowl haematozoa were genetically differentiated from haematozoa infecting other avian orders, except for 1 haematozoan lineage that was shared between waterfowl and perching birds.

Plasmodium spp. infected a wide range of waterfowl from different wetlands, with some strains (particularly lineage 'A' in Fig. 2) appearing to be both generalist and ubiquitous and other lineages (e.g., 'D' in Fig. 2) appearing to be more localized and species specific. Larger sample sizes will be required to adequately determine levels of host specialization by haematozoan species, but our initial results suggest that different species of *Plasmodium* may cover the spectrum from specialist to generalist (Beadell *et al*. 2009; Hellgren *et al*. 2009). *Haemoproteus* spp. appeared subjectively to show greater host specificity than *Plasmodium* spp. Studies in West Africa and elsewhere (Beadell *et al*. 2004, 2009; Bensch *et al*. 2009) have found that *Haemoproteus* spp. generally show more host specificity than *Plasmodium* spp. Beadell *et al*. (2004) found compelling evidence that *Plasmodium* spp. can parasitize multiple host families while *Haemoproteus* spp. species tend to be more specialized. However, our results indicate that some *Plasmodium* spp. specialize on particular hosts within particular avian orders. For example, although lineages A and B infected a wide range of waterfowl hosts, these species have not been found in other avian orders.

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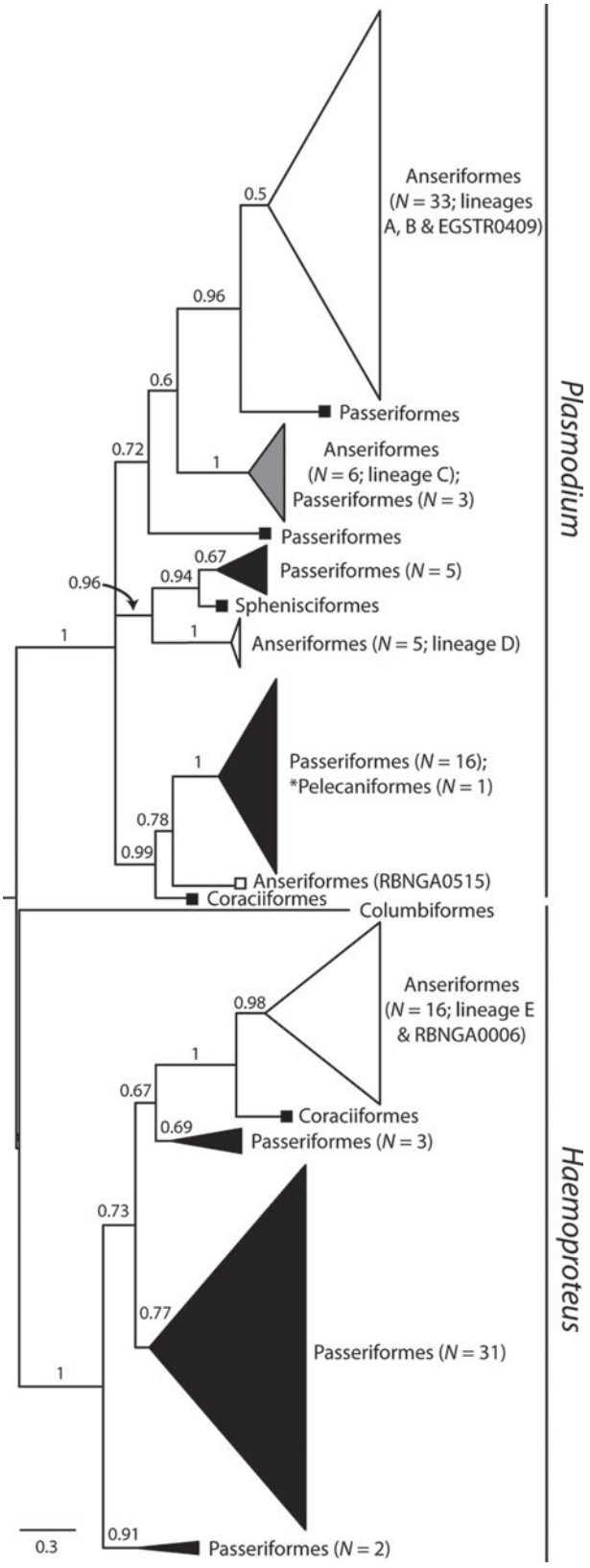

Fig. 4. Bayesian phylogeny of 128 mtDNA cytochrome *b* haplotypes isolated from southern African birds (pinvar = 0·29, alpha = 0·11). Monophyletic clades for Anseriformes isolates (white triangles) and Passeriformes (black triangles) were collapsed for clarity (the asterisk (*) indicates one Passeriformes clade that included a haplotype shared with a single isolate from Pelecaniformes). The triangles illustrate the abundance (height) of the clade and the deepest divergence (width) within the clade. One clade (grey triangle) contained *Plasmodium* spp. isolated from both Anseriformes and Passeriformes, including a haplotype that was shared between these orders. Numbers above branches indicate posterior nodal support.

A recent study on passerines in the western Cape (Schultz *et al*. 2011) suggested a higher overall prevalence and a higher diversity of avian haemosporidia in passerines than found in waterfowl in this study. Schultz *et al*. (2011), however, based species identification on visual identification from gametocyte morphology and their results are thus difficult to compare directly to ours. Their samples were dominated by *Leucocytozoon* spp., with *Plasmodium* spp. infections detected in only 3·2% of sampled birds; we found a much higher incidence of *Plasmodium* spp.

The lack of a seasonal trend within our data was surprising, given that outside the winter rainfall region we would expect to find strong declines in mosquito and culicoides (biting midge) abundance during the drier and colder periods of the year (Altizer *et al*. 2006). Other studies on avian malaria have also found a similar lack of a seasonal or annual trend in infection prevalence (e.g., Bensch *et al*. 2007, although this study misses some annual variation), which invites speculation as to the extent of the influence of vector population dynamics. Although no data on vector population abundance were collected for this study, it would be interesting to determine whether their inclusion would explain variation in avian malaria prevalence. It is possible that, as with avian influenza, a combination of long-distance movement by host species and high levels of variation in infection rates swamp any seasonal influences (Cumming *et al*. 2011). Alternatively, although temperature and photoperiod vary synchronously across the region (with a routinely cold June/July and warm December/January), temperature differences are not always mirrored by differences in rainfall. Given that malaria prevalence may be driven by the covariance between temperature and rainfall (Hay *et al*. 2002), a more sophisticated climate matching procedure to fully eliminate the hypothesis of a rainfall-driven 'malaria season' is needed. Also of relevance on this theme is that little is known about latency and the expression of malaria in waterbirds; it is possible that hyponozoites may remain in the liver for long periods as they do in humans (e.g., White, 2011), confusing our understanding of seasonal transmission dynamics.

Taken together, our results suggest that haematozoa species in southern Africa tend towards being generalist but localized parasites of waterfowl, rather than ubiquitous host specialists. More extensive sampling of our focal species will be needed before we can determine whether any particular strains of haematozoa are linked conclusively to particular

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locations or regions. Our initial results are nonetheless promising, in that they imply that analysis of the biogeography and host use of avian malaria may provide a workable model system for understanding how spatial variation in waterfowl community composition and movement patterns influences their susceptibility to parasitic infections.

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