

**Migration strategy and pathogen risk: non-breeding distribution drives
malaria prevalence in migratory waders**

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Running head: Wader migration and malaria prevalence

29 **Abstract**

30 Pathogen exposure has been suggested as one of the factors shaping the myriad
31 of migration strategies observed in nature. Two hypotheses relate migration
32 strategies to pathogen infection: the “avoiding the tropics hypothesis” predicts
33 that pathogen prevalence and transmission increase with decreasing non-
34 breeding (wintering) latitude, while the “habitat selection hypothesis” predicts
35 lower pathogen prevalence in marine than in freshwater habitats. We tested
36 these scarcely investigated hypotheses by screening wintering and resident
37 wading shorebirds (Charadriiformes) for avian malaria blood parasites
38 (*Plasmodium* and *Haemoproteus* spp.) along a latitudinal gradient in Australia.
39 We sequenced infections to determine if wintering migrants share malaria
40 parasites with local shorebird residents, and we combined prevalence results
41 with published data in a global comparative analysis. Avian malaria prevalence
42 in Australian waders was 3.56% and some parasite lineages were shared
43 between wintering migrants and residents, suggesting active transmission at
44 wintering sites. In the global dataset, avian malaria prevalence was highest
45 during winter and increased with decreasing wintering latitude, after controlling
46 for phylogeny. The latitudinal gradient was stronger for waders that use marine
47 and freshwater habitats (marine+freshwater) than for marine-restricted species.
48 Marine+freshwater wader species also showed higher overall avian malaria
49 parasite prevalence than marine-restricted species. By combining datasets in a
50 global comparative analysis, we provide empirical evidence that migratory
51 waders avoiding the tropics during the non-breeding season experience a
52 decreased risk of malaria parasite infection. We also find global support for the
53 hypothesis that marine-restricted shorebirds experience lower parasite
54 pressures than shorebirds that also use freshwater habitats. Our study indicates
55 that pathogen transmission may be an important driver of site selection for non-
56 breeding migrants, a finding that contributes new knowledge to our
57 understanding of how migration strategies evolve.

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62 **Key words:** Avian malaria, avoiding the tropics, comparative analysis,

63 *Haemoproteus*, latitudinal gradient, migration strategies, *Plasmodium*, shorebirds

64 **Introduction**

65 Identifying the factors that cause variation in migration strategies across
66 populations and species is a central goal in ecology and evolution (Gunnarsson et
67 al. 2005, Marra et al. 2015, Shoji et al. 2015). Parasite exposure is hypothesised
68 to be a driver of migration strategies for a diversity of animal hosts, with
69 behaviours or distributions that reduce exposure to parasites thought to convey
70 an adaptive advantage (Altizer et al. 2011, Satterfield et al. 2015).

71 Haemosporidian malaria parasites are vector-borne blood parasites that infect
72 vertebrates across the globe (Clark et al. 2014, Clark et al. 2015a, Martinsen et al.
73 2008, Moens and Pérez-Tris 2015, Schall 2000). For vector-transmitted parasites,
74 variation in vector encounter rate is a strong determinant of infection risk (Gager
75 et al. 2008, Giles et al. 2014). For instance, saline habitats and low minimum
76 temperatures are considered to be harsh for vector survival and malaria parasite
77 development, leading to reduced malaria prevalence (Figuerola 1999). This
78 spatial variation in malaria prevalence and transmission is predicted to be an
79 important determinant of life-history traits (Lutz et al. 2015), including
80 migratory behaviours (Figuerola and Green 2000).

81 Avian haemosporidians (*Plasmodium* and related *Haemoproteus* spp.;
82 henceforth referred to as ‘avian malaria’) have received increased attention
83 recently as they are globally distributed and can act as selective agents by
84 reducing host fecundity or causing population declines (Lachish et al. 2011,
85 Palinauskas et al. 2011, Valkiūnas 2005). Moreover, molecular techniques allow
86 for tests of geographical structuring in avian malaria phylogenies that can shed
87 light on areas where transmission occurs for migratory species (Hellgren et al.
88 2007, Roos et al. 2015). However, the interplay between migration strategies and

avian malaria prevalence is understudied, primarily owing to a lack of phylogenetically controlled comparative analyses from widespread host groups (Møller and Szép 2011).

Some of the most impressive avian migrations are exhibited by waders (Charadriiformes), with species and populations travelling up to 22,000km on trans-equatorial migrations (Gill et al. 2009). Migratory strategies vary dramatically, from species that use both freshwater and saline habitats during the non-breeding season to species that use marine coastlines exclusively (Piersma 1997, 2007). Wader migrations also vary within species, with some populations wintering in low-latitude tropical areas while others either extend trans-equatorial migrations to more southern latitudes or remain in temperate northern latitudes to winter ("avoiding the tropics"; Nebel 2007). This variation in non-breeding habitat and latitude use likely exposes migratory wader populations to different malaria parasite pressures through variation in vector exposure (Piersma 1997, Yohannes et al. 2009).

Hypothetical impacts of wader migration strategies on malaria parasite risk

Several hypotheses are proposed for how parasite pressure might vary across migration strategies (Altizer et al. 2011, Piersma 1997). Based on the dichotomy of wader distributions, Piersma (1997) formulated the "habitat selection hypothesis" to suggest that species using a broader range of habitats (i.e. using freshwater and marine habitats) are exposed to increased risk of parasite infection. Avian malaria parasites are a tractable model to test this hypothesis, as prevalence can vary across habitats due to climatic variation and vector distributions (Loiseau et al. 2013, Santiago-Alarcon et al. 2012, Sehgal 2015). We

114 therefore expect marine-restricted waders to have lower malaria prevalence
115 compared to species that use freshwater and marine habitats (henceforth
116 marine+freshwater species).

117 In addition to habitat, the use of different wintering latitudes may
118 influence parasite exposure. It is suggested that migratory waders have reduced
119 parasite infections by using high-latitude breeding sites that remain outside of
120 transmission areas (Piersma 1997). Malaria parasite development is impacted by
121 temperature (Beier 1998), and several studies indicate the prevalence, diversity
122 and transmission of avian malaria are higher in tropical than in temperate
123 regions (Clark et al. 2014, Loiseau et al. 2013, Merino et al. 2008, but see
124 Svensson-Coelho et al. 2014). Because of this low transmission at high latitudes,
125 migratory waders are thought to acquire vector-borne infections predominately
126 at stopovers and non-breeding sites (Piersma 1997, Mendes et al. 2005). If so, we
127 expect that infected wintering migrants will carry similar parasite lineages to
128 local residents, supporting the suggestion that transmission can occur in the
129 non-breeding season. Avoiding the tropics could also benefit non-breeding
130 migrants if the costs of migrating farther past the tropics are offset by low
131 parasite pressure. We therefore expect parasite prevalence to be lower in
132 populations “avoiding the tropics” by wintering at more southern latitudes.

133 Molecular studies have examined malaria prevalence for select wader
134 species with different habitat distributions (Yohannes et al. 2009) or within a
135 single flyway (D'Amico et al. 2014, Mendes et al. 2005, Pardal et al. 2014).
136 However, the “avoiding the tropics hypothesis” remains untested due to a lack of
137 sampling from wintering birds across a latitudinal gradient. In contrast,
138 preliminary support for Piersma’s “habitat selection hypothesis” was provided

by Mendes *et al.* (2005), who found significantly higher malaria prevalence in freshwater compared to marine habitats across a narrow latitudinal range in Africa. However, the authors did not distinguish between migrants and year-round residents and, more crucially, the authors deemed their data unsuitable for a phylogenetically controlled analysis due to uneven sampling across habitats (Mendes *et al.* 2005). Comparative analyses control effects of evolutionary history when testing predictions across species (Pagel 1994), and using such methods can ensure that differences in avian malaria pressure across habitats are not simply a by-product of shared evolutionary histories (Møller and Szép 2011).

Here we test the “habitat selection” and “avoiding the tropics” hypotheses using a global comparative analysis of malaria prevalence in waders. We provide data from the previously unsampled East Asian-Australian Flyway by screening resident and wintering waders for parasite infection along a latitudinal gradient in Australia. We use phylogenetic analysis of parasite infections to identify potential transmission areas under the prediction that infected wintering waders will share lineages with local sedentary shorebirds, supporting the suggestion for non-breeding parasite transmission. We then combine our results with published data to generate a global database of wader malaria prevalence. Focussing on migratory species, we use phylogenetic comparative analyses to test two predictions: i) “habitat selection”: predicts marine-restricted migratory species will exhibit lower prevalence than marine+freshwater species; ii) “avoiding the tropics”: predicts decreased prevalence with increasing wintering latitude.

Methods

Australian sampling and molecular methods

Waders were captured using mistnets or cannon nets in Australia in five sampling regions (nine individual sites) across a latitudinal gradient during wintering seasons in 2012 and 2013 (November to March; see Appendix S1 in Supporting Information for species and sample site details). Sampling was concentrated around high-density sites ranging from 17°S (Broome, Western Australia) to 40°S (King Island, Tasmania; Fig. 1, Appendix S1). A blood sample was taken from each bird from the leg or brachial vein and stored as packed red blood cells in ethanol or whole blood in lysis buffer. For birds sampled in southeast Queensland, we also collected thin blood smears to identify whether infections exhibited parasite gametocytes, which would indicate that waders act as “true” hosts for that particular parasite strain (Clark and Clegg 2015, Valkiūnas et al. 2009). DNA was extracted using ammonium acetate/ethanol precipitation and birds were molecularly sexed following Griffiths *et al.* (1998) to confirm extraction quality. Samples were screened for malaria DNA (*Plasmodium* and *Haemoproteus* spp.) using nested PCR to amplify the parasite cytochrome-*b* gene (*cyt-b*), following protocols in Waldenström *et al.* (2004). We used individual flip-cap PCR tubes to prevent contamination, and we included four positive and two negative controls in each run of 16 samples. All samples were screened twice to further minimise false positives and false negatives. None of the negative controls produced amplifications. DNA sequencing was carried out at Macrogen Inc. (Seoul, South Korea) and sequences were compared to the MalAvi database (<http://mbio-serv2.mbioekol.lu.se/Malavi/>) to determine if they were new lineages. For samples with mixed infections (carrying both

Plasmodium and *Haemoproteus*), we re-amplified parasite DNA using genus-specific primers (Clark and Clegg. *in prep*).

Global dataset of avian malaria prevalence in waders

We collated a global dataset by combining Australian results with published studies that molecularly screened waders for malaria. We first searched a database of studies that used PCR to screen for malaria in wild birds to November 2012 (Clark et al. 2014). To identify more recent studies, we searched Web of Science, Google Scholar and PubMed following the protocols listed in Clark *et al.* (2014). Screening of organ tissues can yield important information on the presence of latent parasite infections. However, as the majority of studies only screened blood, we excluded results from organ screening to prevent bias in prevalence estimates (Mendes et al. 2013).

We recorded the following from each site where waders were sampled: absolute latitude, malaria prevalence and total sample size for each species. For each species sampled, we recorded residency as either sedentary (no separation between wintering and breeding grounds) or migratory (spatial separation between wintering and breeding grounds) and in the case of migrants, we recorded stage of annual cycle at time of sampling (breeding, migrating or wintering). In all cases, authors explicitly presented migration status in either the main text or supplementary files (see D'Amico and Baker 2010, Pardal et al. 2014). We used species distribution maps to record whether species are restricted to marine habitats (marine) or use both marine and freshwater habitats during the non-breeding season (BirdLife International 2012).

214 *Phylogenetic reconstruction*

215 We created a molecular wader phylogeny to account for non-independence of
216 species relatedness. We included species in the molecular alignment if they had
217 at least one sequence from the protein coding mitochondrial genes cytochrome
218 oxidase I and *cyt-b* (see Appendix S2 in Supporting Information for GenBank
219 accession numbers). Sequences were aligned in Genious v5.4 (Drummond et al.
220 2011) and the gene alignments were concatenated into a single alignment with a
221 total length of 3688 bp. We used BEAST version 1.6.1 (Drummond and Rambaut
222 2007) to construct the phylogeny with an uncorrelated lognormal relaxed clock
223 and a Yule prior. We generated two runs of 10 000 000 generations with
224 sampling at every 10 000 generations, and used TRACER version 1.6 (available at
225 <http://tree.bio.ed.ac.uk/software/tracer/>) to assess convergence. We removed
226 10% burn-in from each run and combined runs using LogCombiner version 1.4.7
227 (Drummond and Rambaut 2007). The phylogeny included 39 of the 46 species in
228 the final dataset. We grafted the remaining species onto the most appropriate
229 nodes of the final consensus tree as polytomies using the latest phylogenetic
230 information for Charadriiformes (Baker et al. 2007, Mayr 2011). As the resulting
231 tree was not ultrametric, equal branch lengths were set using Grafen's method.
232 The wader phylogeny is presented in Appendix S3.

233 We constructed molecular phylogenies from parasite *cyt-b* sequences
234 recovered in Australian samples using identical protocols to the methods
235 outlined above for waders. To investigate whether parasite phylogenies could
236 yield information about where infections were acquired, we included all
237 published avian malaria sequences recovered in Australia and Southeast Asia
238 (see Clark et al. 2015b for details on parasite sequence acquisition). In addition,

we included parasite sequences previously recovered from waders and other non-passerine shorebirds (Levin et al. 2011, 2012, Mendes et al. 2005, 2013) to investigate whether waders in Australia carry similar lineages to shorebirds around the world. Separate phylogenies were constructed for *Plasmodium* and *Haemoproteus* spp.

Statistical analyses

All analyses were carried out in R, version 3.0.1 (R Development Core Team 2008). We tested for effects of explanatory variables on malaria prevalence in a comparative analysis. We used Bayesian phylogenetically controlled generalised linear mixed models (pGLMMs) in the package ‘mcmcGLMM’ (Hadfield 2010). The binomial response variable in pGLMMs weights for sample size by including numbers of infected and uninfected individuals for each species. We used weak proper priors with a low degree of belief for all parameters (variance > 0, degree of belief $\nu = 1$). Models were run for 11 000 000 iterations with a burn-in of 100 000 and a thinning value of 1 000. Significance of terms was inferred if the 95% posterior confidence interval for the coefficient (γ) did not overlap with zero and if P_{MCMC} -values were equal to or less than 0.05. We tested all possible combinations of terms and interactions and used deviance information criterion (DIC) to select final models.

Too few individuals from sedentary species were available for inclusion of a ‘residency’ term in pGLMMs (N = 305). Sedentary species were therefore excluded from analyses and are only included in descriptions of overall prevalence and identification of parasite sequences (see Results). Using only migrants, the dataset for analyses comprised of 3422 individuals (from 36

species). Additional stressors related to long flights and high densities may result in different disease pressures depending on the stage of the annual cycle (i.e. Breeding vs Migrating vs Wintering; Buehler and Piersma 2008). We therefore fitted a pGLMM with habitat (two levels: marine or marine+freshwater) and migration stage (three levels: breeding, migrating or wintering). We also included mean body mass for each species (from Dunning Jr 2007) as a covariate. We tested all combinations of interactions during model selection. Due to low sample sizes for some species (Appendix S1), we tested the validity of our conclusions by excluding all species with fewer than 15 observations and repeating analyses. This reduced dataset comprised of 3236 individuals from 19 migratory species.

To investigate the “avoiding the tropics” hypothesis, we tested for a latitudinal gradient in wintering malaria prevalence using a pGLMM that only included wintering migrants (N = 1730 individuals; 27 species). Explanatory variables were absolute latitude, habitat, body mass and all interactions. Absolute sampling latitude and body mass were natural log transformed to reduce the influence of extreme values.

While we obtained sequences from 29 of 40 infections from Australian waders (see Results), we did not perform separate analyses for individual parasite genera because most studies published to date have not produced an adequate number of lineages for genus-specific analysis (e.g. Mendes et al. 2005 sequenced seven of 54 infections and recovered a total of three lineages).

Results

Avian malaria prevalence in Australian waders

We sampled 1125 waders in Australia, 54 of which were from low latitudes (17° - 19°S), 212 from mid latitudes (27° - 28°S) and 859 from high latitudes (38° - 40°S; Fig. 1, Appendix S1). Samples were taken from 19 species, with 15 species sampled as wintering migrants (Appendix S1). We detected 40 malaria infections for a prevalence of 3.56% (including migratory and sedentary species). When sites were grouped by latitude category, there was a trend of higher prevalence in low latitudes (13.0%) compared to mid- and high-latitudes (3.3% and 3.0%, respectively), although sample sizes in low latitudes prevented robust comparisons to detect a latitudinal gradient (either for migrants alone or for the full dataset of migratory + sedentary species) due to inadequate mixing of MCMC chains. Prevalence in marine+freshwater migrants was 3.0% (N=300 individuals; 5 species) and 3.9% in marine-restricted migrants (N=665 individuals; 9 species), though the vast majority of marine+freshwater individuals were sampled in mid- and high-latitudes where overall prevalence was low (Appendix S1).

We recovered 13 malaria *cyt-b* lineages from 29 sequenced infections (Table 1). The remaining 11 infections failed to produce clean sequences due to weak amplifications. Two infections were mixed, containing both *Plasmodium* and *Haemoproteus* parasites. The full, un-collapsed parasite phylogenies are presented in Appendix S4 (Figs. A1 and A2). From 18 sequenced *Haemoproteus* infections, we recovered four *cyt-b* lineages (Table 1; Fig. 2). For *Plasmodium* spp., we recovered nine lineages from 13 infections (Table 1; Fig. A2). False positives were unlikely, as none of the sequences matched sequences from our positive controls and seven of the 13 lineages recovered were new records for GenBank and MalAvi. Stained blood smears were available for seven of the

infected birds (representing six lineages), and parasite gametocytes were observed in all seven smears (Table 1).

The four *Haemoproteus* lineages were all new, and two of these were recovered from both migratory and resident waders in Australian wintering sites (RUDTURN01 and SDLG01; Table 1; Fig. 2). Lineage SDLG01 was found in both freshwater and marine-restricted migratory species that co-occur at wintering sites (Table 1). Two sub-genera are currently recognised for *Haemoproteus* (*Haemoproteus* and *Parahaemoproteus*; Valkiūnas 2005), and the four *Haemoproteus* lineages grouped into a well-supported clade with other non-Passerine waterbird lineages (Pelicaniformes and Charadriiformes hosts) in the *Parahaemoproteus* clade (Fig. 2; Fig. A1). Four of the nine *Plasmodium* lineages recovered in migratory waders have also been found in Australian resident passerines (Table 1). In contrast to *Haemoproteus*, the *Plasmodium* lineages did not group together and instead were spread across the phylogeny (Fig. A2).

Global avian malaria prevalence in migratory waders

The global dataset, including Australian samples, comprised of 3727 sedentary and migratory waders from 46 species (Appendix S1). Sampling ranged from latitudes 73°N to 53°S across five continents (North America, South America, Africa, Europe, and Australia). The majority of samples were from migrants, with only 305 samples from sedentary waders (Appendix S1). Among migrants, 1730 samples were from wintering birds (27 species; sampled from 53°N to 40°S), 1237 from migrating birds (13 species; sampled from 53°N to 38°N) and 416 from breeding birds (12 species; sampled from 73°N to 71°N). Global malaria

prevalence (including sedentary and migratory waders) was relatively low and variable among species (mean $6.2 \pm 1.6\%$; Appendix S1).

Influence of phylogeny and migration strategies on avian malaria prevalence

The global dataset provided robust power for comparative analyses (MCMC chains exhibited adequate mixing and autocorrelations for posterior samples were all less than 0.1). We found a strong influence of phylogeny ($\gamma = 8.55$; 95% CI = 1.24, 21.06), an effect that was partially driven by similar habitat and migration strategies for some related species (e.g. both *Limosa* spp. are migratory marine species, *Erythronyx cinctus* and both *Vanellus* spp. are all resident freshwater species, and four of five species in the *Himantopus*/*Haemotopus*/*Recurvirostra* clade are freshwater users; Appendices S1 and S3). However, this phylogenetic effect was also partially due to similarities in malaria prevalence for some related species. Infections were found in 10 of 13 species in the well-sampled *Calidris*/*Arenaria*/*Philomachus* clade (N=2815 individuals), with five of the seven marine+freshwater species and three of the six marine species exhibiting higher prevalence than the mean for their respective habitat distributions (Appendices S1 and S3). In contrast, no infections were found in the three *Pluvialis* species, though the total sample size for this genus was 18 individuals total.

After accounting for phylogeny, the best model for the migrant dataset included stage of annual cycle and habitat strategy as significant terms. Wintering birds had significantly higher prevalence (mean $8.5 \pm 2.5\%$) than either migrating or breeding birds (mean $0.7 \pm 0.6\%$ vs $0.3 \pm 0.2\%$, respectively; $\gamma = 4.19$; 95% CI = 0.53, 8.14; $P_{MCMC} = 0.01$; Fig. 3a). We found global

comparative support for Piersma's "habitat selection hypothesis", with marine-restricted species exhibiting significantly lower malaria prevalence than marine+freshwater species (mean $2.3 \pm 0.9\%$ vs $5.9 \pm 1.7\%$, respectively; $\gamma = -2.04$; 95% CI = -4.03, -0.03; $P_{MCMC} = 0.04$; Fig. 3b). When excluding species with fewer than 15 samples, MCMC chains mixed adequately and effect sizes for migration stage and habitat were smaller but were still significant (see Appendix S5 in Supporting Information for model statistics).

Avoiding the tropics: a latitudinal gradient in avian malaria prevalence

When considering only wintering migrants, phylogeny again had a strong influence ($\gamma = 11.05$; 95% CI = 1.47, 27.35) and the best model included significant terms for habitat and sampling latitude (Appendix S5). Malaria prevalence decreased significantly with increasing wintering latitude ($\gamma = -6.48$; 95% CI = -11.22, -1.77; $P_{MCMC} = 0.001$) and was significantly lower in marine than marine+freshwater species ($\gamma = -18.05$; 95% CI = -37.00, -0.01; $P_{MCMC} = 0.04$). An interaction between latitude and habitat reduced model deviance and was borderline significant ($\gamma = 5.53$; 95% CI = -0.19, 11.94; $P_{MCMC} = 0.053$). Model predictions indicate the latitudinal gradient was stronger for marine+freshwater species (Fig. 4). Body mass and all other interactions did not significantly influence prevalence or improve model deviance. Results were similar when excluding species with low sample size, although P_{MCMC} values for habitat and the habitat*latitude interaction were marginally non-significant ($P_{MCMC} = 0.06$ for each effect, Appendix S5).

Discussion

Using a global dataset that includes the previously unsampled Australian flyway, we present the first evidence that waders “avoiding the tropics” during winter experience a decreased risk of malaria parasite infection. The latitudinal gradient was stronger for marine+freshwater species, suggesting that wintering farther from the tropics may not benefit all populations equally. We also provide comparative evidence that species using both marine and freshwater habitats have higher parasite prevalence than marine-restricted species, supporting Piersma’s “habitat selection hypothesis”. Migratory populations with different non-breeding distributions may be exposed to different interspecific interactions, a finding that has implications for our understanding of how migration strategies evolve.

High malaria prevalence in winter: implications for avoiding the tropics

Our results show that waders experience higher malaria burdens in winter compared to migration and breeding periods. We also found that migrants are carrying a diversity of parasite lineages, some of which are shared with residents in the wintering range. Indeed, six of the 13 recovered parasite lineages have also been found in Australian resident birds, indicating that these lineages can be actively transmitted on the wintering grounds. Although only seven infected waders had corresponding blood smears, all seven showed parasite gametocytes and were therefore confirmed as true infections that were transmissible to vectors. Our results support the suggestion that long-distance migrants may be susceptible to infections following arrival to wintering grounds, perhaps because their immune function can be compromised from migration-induced fatigue or muscle injury (Klaassen et al. 2000, Piersma 1997). Moreover, the recovery of a

well-supported *Haemoproteus* clade that is only currently known to infect shorebirds, including two lineages that were shared between migratory and resident waders, suggests that migrants play a role in shaping the population dynamics of these parasites. In contrast to *Haemoproteus* spp., *Plasmodium* lineages in Australasia are typically host-generalists with wide geographic ranges (Clark et al. 2014, Clark and Clegg 2015, Clark et al. 2015b), making it difficult to determine where transmission occurred. For instance, *Plasmodium* lineages ORW1 and EMSP006 have both been found infecting resident birds throughout Australasia (Huang et al. 2015, Ishtiaq et al. 2007), and so these parasites may have been picked up either during a migratory stopover or after arrival in Australia. The presence of latent infections could also be problematic for discovering transmission areas, as parasites can be missing from the blood and will not be amplified during PCR. However, our conclusion that winter represents an increased risk of infection does not hinge on distinguishing between recently acquired and re-activated infections. Latent infections cannot be picked up by a vector, and so the fact that active infections are more abundant in winter is good evidence that wintering birds face higher parasite pressures due to an increased chance of infections being passed around the population (Begon et al. 2002).

We also find evidence that increased wintering latitude results in decreased malaria prevalence, supporting our prediction that populations that ‘avoid the tropics’ experience decreased risk of infection. A similar trend was observed for tundra swans in Alaska, with populations that spend more time near the tropics exhibiting increased malaria prevalence (Ramey et al. 2012). We propose that shorebird populations wintering farther from the tropics are

exposed to fewer disease-carrying vectors, as both vector diversity and the rate of within-vector parasite development are reduced in high latitudes (Loiseau et al. 2013, Rueda 2008). Because pathogen infection can reduce body condition and delay arrival times (Bradley and Altizer 2005, Møller et al. 2004), risk of infection may place additional pressures on migrants to choose the best non-breeding sites. Collectively, our findings support a broader recognition that choosing a non-breeding site is an important and complex behavioural trait that might have impacts throughout the life cycle (Gunnarsson et al. 2005, Marra et al. 2015, Shoji et al. 2015).

Support for Piersma's habitat selection hypothesis

Our results also support the hypothesis that marine-restricted waders are using habitats with relatively low malaria parasite pressures (Mendes et al. 2005, Piersma 1997, Yohannes et al. 2009). Moreover, our study is the first to highlight a difference in parasite prevalence in wintering birds with different habitat choices, as marine species showed a weaker latitudinal gradient in wintering malaria prevalence than marine+freshwater species. Although the vectors that transmit avian malaria are poorly studied (Lima and Bensch 2014), evidence increasingly suggests that most vectors rely on permanent or semi-permanent freshwater to breed (Kalluri et al. 2007, Njabo et al. 2009, Santiago-Alarcon et al. 2012, Valkiūnas et al. 2015). The higher incidence of malaria infections in marine+freshwater wader species implicates the availability of vector breeding sites as a strong determinant of parasite risk. For freshwater habitats, changes in latitude may correlate with changes in climate that can influence the prevalence and diversity of parasites (Oakgrove et al. 2014, Sehgal et al. 2010). Climate

influences may be less apparent for marine habitats, where lack of freshwater and high salt spray will be similarly unfavourable to vectors at different latitudes (Figuerola 1999). Consequently, marine-restricted species may minimize overall parasite exposure by migrating between Arctic breeding areas and coastal habitats (Alerstam et al. 2003), and could experience fewer disease-related benefits from avoiding the tropics than marine+freshwater species. Nevertheless, our comparison of latitudinal trends for marine and freshwater species was not statistically significant and so should be interpreted with appropriate caution.

Influence of phylogeny on malaria parasite prevalence in waders

In their study of the “habitat selection hypothesis”, Mendes *et al.* (2005) were unable to use a comparative analysis, a limitation that was recognised by the authors and was highlighted by Møller & Szép (2011). While we encountered similar problems analysing the Australian subset, our global analysis suggests that evolutionary history can influence malaria prevalence for some species. Infections were commonly found in species belonging to the *Calidris/Arenaria/Philomachus* clade, with the majority of species showing above average prevalence for their respective habitats. While it is unclear if certain shared traits predispose species to malaria, it is possible that this high prevalence is due to microhabitat use or foraging characteristics that expose species to high vector densities. For instance, the marine-restricted *Arenaria interpres* regularly scavenges around rotting organic material and may experience unusually high pathogen exposure (Mendes et al. 2006), while *Philomachus pugnax* often uses rice fields in Africa that remain wet and could adequately maintain vectors during the dry winter season (Mendes et al. 2013).

Alternatively, closely related species could have similar immune strategies that allow them to better tolerate malaria infections, though this idea warrants future study.

Importance of sample size and the biological significance of malaria in waders

Although comparative pGLMMs weight for samples size and our results were robust when excluding poorly sampled species, we stress the importance of large sample sizes for parasite screening in wildlife. Despite extensive sampling, migratory marine+freshwater species were represented by only 300 samples from five species in Australia, the vast majority of which were sampled at high latitudes where prevalence is likely to be lower. Monitoring for natural prevalence levels is crucial in disease ecology (Clark et al. 2015b, Grogan et al. 2014), and sample size is an important consideration for identifying populations at risk.

The influence of animal dispersal on disease incidence has gained increased recognition of late (Altizer et al. 2011, Bauer et al. 2015, Clark and Clegg 2015, Cohen et al. 2015, Jenkins et al. 2012, Møller and Szép 2011, Ramey et al. 2012). Some migration strategies are thought to be the result of species actively avoiding parasite-rich habitats (Piersma 1997, Qviller et al. 2013, Sandström et al. 2013), suggesting that migrants must balance the costs of extra migration distances with the potential benefits of reduced pathogen exposure. Despite the low overall prevalence in our dataset, we found that nearly 10% of waders wintering in low latitude sites were infected with malaria across the globe. We propose that malaria in waders may be more common than previously supposed (Greiner et al. 1975), indicating that these parasites could have

important but overlooked biological significance. However, the low overall parasite prevalence in our study raises questions about how important malaria parasites are for shaping migratory patterns in waders. A prevalence of 10% in tropical sites may not be adequate to act as a strong selective pressure and might not outweigh the added benefits of wintering in the tropics, such as the reduced flight distances (Klaassen 1996, Somveille et al. 2015). Moreover, we have no conclusive evidence for pathological effects of malaria parasites in shorebirds (but see Mendes et al. 2013), and it seems likely that any impacts might differ between *Plasmodium* and *Haemoproteus* parasites (Valkiūnas 2005). A multiplicity of variables can shape migration patterns in birds (Bauer et al. 2010, Duriez et al. 2012, Klaassen et al. 2000, 2012), and the relative importance of malaria parasites compared to these factors as drivers of migratory routes requires further consideration.

Data Accessibility

To be completed nearer to publication

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Additional Supporting Information may be found in the online version of this article:

Appendix S1: Sampling locations and haemosporidian prevalence for waders in the final global dataset.

Appendix S2: GenBank accession numbers for sequences used in the wader phylogeny.

Appendix S3: Molecular wader phylogeny used for phylogenetic MCMCglms.

Appendix S4: Full molecular parasite phylogenies for *Haemoproteus* and *Plasmodium* parasite lineages.

Appendix S5: Model statistics for initial and final pGLMMs.

774 Table 1: Avian malaria parasite lineages recovered from waders in Australia. Also presented are the known geographic ranges of
775 lineages based on comparison to the avian malaria database, MalAvi (<http://mbio-serv2.mbioekol.lu.se/Malavi/>). * indicate lineages
776 that had corresponding blood smears and were confirmed to exhibit gametocytes, suggesting complete parasite development within the
777 host.
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Lineage	Parasite genus	GenBank accession	Number of infections	Migratory wader hosts	Australian resident hosts	Countries where malaria lineage occurs
BTGOD01*	<i>Plasmodium</i>	KT777732	3	<i>Limosa lapponica</i> , <i>Tringa brevipes</i> , <i>Arenaria interpres</i>	none	Australia
EMSP006	<i>Plasmodium</i>	AB741487	1	<i>Calidris ruficollis</i>	none	Australia, China, Japan, South Korea
FANTAIL01	<i>Plasmodium</i>	JX021567	1	<i>Calidris tenuirostris</i>	Passerines	Australia, Czech Republic, Singapore
GRW09	<i>Plasmodium</i>	FJ404701	1	<i>Charadrius leschenaultii</i>	Passerines	Australia, Cameroon, Gabon, Malawi, Netherlands, Nigeria, Russia, Sweden, Switzerland, Tanzania, Ukraine
GRW15	<i>Plasmodium</i>	DQ368380.1	2	<i>Charadrius leschenaultii</i> , <i>Recurvirostra novaehollandiae</i>	Passerines	Australia, Sweden
ORW1*	<i>Plasmodium</i>	AY714203	1	<i>Limosa lapponica</i>	Passerines	Australia, India, Japan, Myanmar, Papua New Guinea, Russia, United Kingdom
RHIALB02	<i>Plasmodium</i>	JX418219	1	<i>Arenaria interpres</i>	none	Australia, Philippines
STNT01*	<i>Plasmodium</i>	KT777740	1	<i>Calidris ruficollis</i>	none	Australia
STNT02*	<i>Plasmodium</i>	XXXX	2	<i>Calidris ruficollis</i> , <i>Tringa brevipes</i>	none	Australia

RUDTURN01	<i>Haemoproteus</i>	XXXX	2	<i>Arenaria interpres</i>	Resident waders	Australia
SANDPLOV01*	<i>Haemoproteus</i>	XXXX	3	<i>Charadrius leschenaultia, Calidris ruficollis</i>	none	Australia
SDLG01	<i>Haemoproteus</i>	XXXX	11	<i>Calidris alba, Calidris tenuirostris, Limosa lapponica, Arenaria interpres</i>	Resident waders	Australia
TATTLR01*	<i>Haemoproteus</i>	XXXX	2	<i>Tringa brevipes, Calidris ruficollis</i>	none	Australia

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FIGURE LEGENDS

Fig. 1: Australian sampling sites (dots) and wader sample sizes (number of birds infected with avian malaria parasites/ total number of birds sampled). Individual sample sites were grouped into five sampling regions based on proximity and overlap of migratory wader flocks.

Fig. 2: Bayesian molecular phylogeny of *Haemoproteus* cytochrome-*b* lineages from the sub-genera *Haemoproteus* and *Parahaemoproteus*. All *Haemoproteus* lineages previously recorded in Australia, China and Southeast Asia were included in the phylogeny. Numbers at nodes indicate Bayesian posterior probabilities. Lineages in black font were recovered from shorebirds in the order Charadriiformes. Lineages in bold were recovered from waders in Australia. Asterisks (*) indicate lineages shared between resident and migratory waders in Australia. COL, Columbiformes; CHD, Charadriiformes; COR, Coraciiformes; PIC, Piciformes; PEL, Pelicaniformes; STR, Strigiformes.

Fig. 3: Avian malaria parasite prevalence in migratory waders (a) at different migratory stages and (b) with different habitat selection strategies. Box and whisker plots indicate third quartiles (hinges of box) and 95% confidence intervals (error bars) of the median prevalence. Fresh = wader species that use freshwater and marine habitats during migration and winter; Marine = wader species that are restricted to marine habitats during migration and winter.

Fig. 4: Avian malaria parasite prevalence across wintering latitudes (natural log transformed) for migratory waders with differing habitat strategies: (a) species that utilise freshwater and marine habitats; (b) species that are restricted to marine habitats. Regression lines are phylogenetically-controlled MCMCglmm predictions with 95% confidence intervals (shaded areas). To improve interpretation, data points are scaled according to sample size.