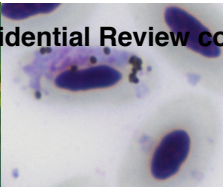


## Co-infections and environmental conditions drive the distributions of blood parasites in wild birds

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24    **Abstract**

- 25        1. Patterns of pathogen co-occurrence can affect the spread or severity of disease.  
26            Yet due to difficulties distinguishing and interpreting co-infections, evidence  
27            for the presence and directionality of pathogen co-occurrences in wild hosts is  
28            rudimentary.
- 29        2. We provide empirical evidence for pathogen co-occurrences by analysing  
30            infection matrices for avian malaria (*Haemoproteus* and *Plasmodium* spp.)  
31            and parasitic filarial nematodes (microfilariae) in wild birds (New Caledonian  
32            *Zosterops* spp.).
- 33        3. Using visual and genus-specific molecular parasite screening, we identified  
34            high levels of co-infections that would have been missed using PCR alone.  
35            Avian malaria lineages were assigned to species level using morphological  
36            descriptions. We estimated parasite co-occurrence probabilities, while  
37            accounting for environmental predictors, in a hierarchical multivariate logistic  
38            regression.
- 39        4. Co-infections occurred in 36% of infected birds. We identified both positive  
40            and negatively correlated parasite co-occurrence probabilities when  
41            accounting for host, habitat and island effects. Two of three pairwise avian  
42            malaria co-occurrences were strongly negative, despite each malaria parasite  
43            occurring across all islands and habitats. Birds with microfilariae had elevated  
44            heterophil to lymphocyte ratios and were all co-infected with avian malaria,  
45            consistent with evidence that host immune modulation by parasitic nematodes  
46            facilitates malaria co-infections. Importantly, co-occurrence patterns with  
47            microfilariae varied in direction among avian malaria species; two malaria

48 parasites correlated positively but a third correlated negatively with  
49 microfilariae.

50 5. We show that wildlife co-infections are frequent, possibly affecting infection  
51 rates through competition or facilitation. We argue that combining multiple  
52 diagnostic screening methods with multivariate logistic regression offers a  
53 platform to disentangle impacts of environmental factors and parasite co-  
54 occurrences on wildlife disease.

55

56 Key words: avian malaria, *Haemoproteus*, heterophil to lymphocyte ratio, filarial  
57 parasite, immune modulation, parasite co-occurrence

58

## 59 **Introduction**

60 How pathogens are distributed and how changing environments cause disease spill-  
61 over across species or geographic barriers are key questions in ecology (Wood *et al.*  
62 2012; Hoberg & Brooks 2015; Plowright *et al.* 2015; Wells *et al.* 2015). While the  
63 environment undoubtedly influences pathogen infections (Budria & Candolin 2013;  
64 Sehgal 2015), hosts often carry multiple pathogens whose interactions can alter  
65 infection dynamics (Cattadori, Boag & Hudson 2008; Johnson & Hoverman 2012).  
66 Infection with one pathogen can increase a host's susceptibility to other pathogens or  
67 to harmful disease (Bordes & Morand 2011). For example, chickens infected with  
68 *Staphylococcus aureus* bacteria develop more severe disease when inoculated with  
69 influenza than those without co-occurring bacteria (Kishida *et al.* 2004). Pathogen  
70 interactions might also be antagonistic. In leaf-cutting ants, competition between  
71 fungal pathogen strains leads to decreased overall pathogen transmission (Hughes *et*  
72 *al.* 2004). Yet while interactions such as competition and facilitation form the

73 foundations of ecology (Dayton 1971), detecting wildlife pathogen associations is  
74 challenging due to (1) difficulties distinguishing co-infections (Valkiūnas *et al.* 2006;  
75 Tompkins *et al.* 2011) and (2) a lack of statistical approaches to disentangle impacts  
76 of environmental predictors (Muturi *et al.* 2008; Fenton *et al.* 2014). Hierarchical  
77 multivariate approaches overcome this hurdle by assessing both environmental  
78 influences and interspecific co-occurrences in joint distribution models (Ovaskainen,  
79 Hottola & Siitonen 2010; Kissling *et al.* 2012). We use one such tool, multivariate  
80 logistic regression, to describe the presence and directionality of blood parasite co-  
81 occurrences in wild birds.

82       Haematozoan blood parasites, including haemosporidians (*Plasmodium* and  
83 *Haemoproteus* spp.; collectively referred to here as ‘malaria’ parasites to avoid  
84 confusing ‘haemosporidian’ and ‘haematozoan’) and microfilaria (blood stages of  
85 filarial nematodes), are vector-transmitted parasites that often exist in co-infection  
86 (Bush 2001; Atkinson, Thomas & Hunter 2008; Clark, Clegg & Lima 2014). Because  
87 both parasites are important disease agents, understanding factors that drive their  
88 transmission and occurrence is vital to unravel their impacts on hosts (Muturi *et al.*  
89 2008; Griffiths *et al.* 2015). Haematozoans are strongly driven by environmental  
90 factors, such as temperature and habitat, that can limit parasite development or vector  
91 distributions (Rogers *et al.* 2002; Santiago-Alarcon, Palinauskas & Schaefer 2012;  
92 Freed & Cann 2013; Sehgal 2015). However, haematozoan infections may also be  
93 influenced by biotic parasite interactions (Su *et al.* 2005; Telfer *et al.* 2010).  
94 Experimental work in mammals shows that parasitic nematodes can modulate  
95 immune responses of hosts by depressing antigen-recognising lymphocytes while  
96 increasing neutrophils, potentially increasing concomitant malaria transmission  
97 (Nacher *et al.* 2001; Graham *et al.* 2005; Su *et al.* 2005; Muturi *et al.* 2008).

98 Competition between malaria strains can also occur and is likely to influence within-  
99 host progression (Bell *et al.* 2006). Yet despite increasing evidence for parasite  
100 associations in model mammalian hosts (Telfer *et al.* 2010; Fenton *et al.* 2014),  
101 evidence from non-model hosts is primarily experimental and remains limited by a  
102 paucity of co-infection data (Jackson *et al.* 2006; Knowles 2011; Tompkins *et al.*  
103 2011).

104 We assess the importance of environmental variables and interspecific  
105 associations on haematozoan parasite occurrences in four avian species (family  
106 Zosteropidae) in New Caledonia. We examine a possible mechanism for within-host  
107 parasite interactions by asking if infections result in altered host immune profiles.  
108 Birds are an ideal study system as avian haematozoans are common and co-infections  
109 are abundant (Sehgal, Jones & Smith 2005; Atkinson, Thomas & Hunter 2008;  
110 Marzal *et al.* 2011; Marzal 2012; Oakgrove *et al.* 2014; van Rooyen *et al.* 2014; Lutz  
111 *et al.* 2015; Goulding *et al.* 2016). In New Caledonia, *Zosterops* spp. are commonly  
112 infected with a diversity of avian malaria parasites (Ishtiaq *et al.* 2010; Olsson-Pons *et*  
113 *al.* 2015). Possible associations between *Zosterops* spp. and filarial parasites have not  
114 been studied.

115 Based on evidence for parasite competition in mammals (Bell *et al.* 2006;  
116 Telfer *et al.* 2010; Hellard *et al.* 2015), we predicted that distinct avian malaria  
117 parasites would exhibit negatively correlated infection probabilities when accounting  
118 for environmental drivers, indicating possible parasite competition. We predicted that  
119 malaria species would positively correlate with microfilaria, based on experimental  
120 evidence that immune-modulating nematodes can facilitate malaria co-infections  
121 (Druilhe, Tall & Sokhna 2005; Su *et al.* 2005).

122

123   **Methods**

124   *Field sampling and laboratory methods*

125   New Caledonia is a sub-tropical Pacific archipelago consisting of four main islands  
126   (Fig. 1a). The archipelago supports four *Zosterops* spp., including the regionally  
127   widespread *Z. lateralis*, the New Caledonian endemic *Z. xanthochrous*, and two  
128   single-island endemics, *Z. minutus* and *Z. inornatus* (both of which only occur on the  
129   island of Lifou; Dutson 2012). All four species are omnivorous passerines that occur  
130   in mixed-species flocks. We captured *Zosterops* spp. with mistnets on the four main  
131   islands from Jan. to March 2014. Sites were chosen to represent the three primary  
132   forested habitats in New Caledonia, namely dry lowland forest (Grand Terre, Ouvéa),  
133   lowland rainforest (Ouvéa, Lifou and Maré) and montane rainforest (Grand Terre; see  
134   supporting information Fig. S1 for site map). Blood samples were collected from each  
135   bird (n = 275). Blood smears were also taken for 245 birds.

136         Avian malaria PCR screening and sequencing followed Clark *et al.* (2015),  
137   with the following variations. Sequences suggested amplification bias towards  
138   *Plasmodium* spp. when co-occurring with *Haemoproteus* spp., with clean  
139   *Plasmodium* sequences (i.e. absence of double peaks) retrieved from 16 confirmed  
140   *Plasmodium*/*Haemoproteus* co-infections (see below for smear screening). Eight  
141   known co-infections produced *Haemoproteus* sequences, while a further six produced  
142   double peaks (re-sequencing of all six producing clean *Plasmodium* sequences).  
143   *Haemoproteus* lineages were therefore characterised using genus-specific primers  
144   designed from sequences recovered in Australasian hosts (Clark & Clegg 2015; Clark,  
145   Clegg & Klaassen 2016). These primers successfully amplified *Haemoproteus* DNA  
146   from all visually observed *Plasmodium*/*Haemoproteus* co-infections. A Bayesian  
147   phylogeny was constructed to estimate malaria relationships, following Clark *et al.*



(2015). For malaria lineages presenting all developmental stages in corresponding single-infection smears, we identified parasites to species (see supporting information for parasite identifications). For microfilaria, we screened samples by amplifying 782bp of the parasite large subunit rDNA. GenBank accessions for parasite lineages are XXXX and XXXX, respectively. Malaria lineages are also deposited in the MalAvi database (Bensch, Hellgren & Pérez-Tris 2009). PCR protocols, phylogenetic methods and the malaria consensus phylogeny (Fig. S2) are presented in supporting information.

The proportion of heterophils (avian equivalent of neutrophils) relative to lymphocytes (heterophil to lymphocyte ratio; H/L) is a reliable indicator of avian immune responses (Davis, Maney & Maerz 2008) and a useful metric to observe whether parasites modulate host immune systems. Because filarial parasites can decrease a host's ability to produce immune cells (lymphocytes in this case; Chatterjee *et al.* 2015) in response to antigens, while also increasing inflammatory neutrophils, we may expect microfilaria infection to lead to increased H/L ratios if such immune modulation occurs in birds. To visually screen for parasites and characterise H/L ratios, we examined blood smears. Smears were fixed in methanol and stained with 10% Giemsa. The entire smear was screened at 200× for microfilaria. We screened at least 100 fields at 1,000× to identify malaria parasites and to calculate H/L ratios by categorising the first 100 white blood cells observed as heterophil, lymphocyte, eosinophil or monocyte.

#### *Analysis of parasite distributions and co-occurrence probabilities*

We combined data with published malaria data from 174 New Caledonian *Zosterops* individuals (Olsson-Pons *et al.* 2015) for a total of 449 birds (Table 1a). The final

dataset included 82 haematozoan co-infections, 16 from published data and 66 from the 2014 data. Note however that observed co-infection occurrences are likely underestimates, as only the 2014 samples were screened with both smears and genus-specific primers. We gathered infection data from four parasite groups (*H. zosteropsis*, *H. killangoi*, *Plasmodium* spp. and microfilaria; see supporting information for descriptions and molecular barcoding of *H. zosteropsis* and *H. killangoi*) across 17 sites [46 birds in montane rainforest (Grand Terre), 111 in open lowland forest (Grand Terre and Ouvéa) and 292 in lowland rainforest (Maré, Lifou and Ouvéa); Fig. 1a; Fig. S1].

In addition to *Zosterops* spp., we included abundance data from other avian species (485 individuals in total) that were also captured across the 17 sites. Host availability can vary such that some hosts are in low abundance in particular habitats, and this variation could influence parasite distributions (Wells *et al.* 2012). Abundance data from additional avian families was therefore used in conjunction with *Zosterops* abundance data to assess the influence of *Zosterops* spp. proportional abundance on parasite occurrences. This parameter is warranted as *Zosterops* spp. are the most common hosts for many New Caledonian avian malaria lineages (Ishtiaq *et al.* 2010; Olsson-Pons *et al.* 2015), indicating that local *Zosterops* abundances could influence transmission (Moens *et al.* 2016; Ricklefs *et al.* 2016). Moreover, *Zosterops* spp. are the only hosts recorded for lineages belonging to the *Haemoproteus* spp. tested here, a pattern supported by morphological data ranging from Africa to Australasia (Valkiūnas 2005). Thus, Zosteropidae hosts likely represent the only available ‘habitat’ for *H. zosteropsis* and *H. killangoi* to asexually develop. *Zosterops* spp. sample sizes ranged from three to 105 and proportional abundance ranged from 19.4 - 100% across sites.

To model individual infection probabilities, we used a hierarchical multivariate logistic regression to decompose variation due to environment (specified by covariates) and interspecific parasite co-occurrences (specified by a variance/covariance matrix). Here, a positive correlation signifies parasites that co-occurred more often than expected by chance given their respective environmental affinities, while a negative correlation signifies the opposite. Note that positive or negative correlations do not necessarily represent explicit within-host parasite interactions, as infection intensity and, ideally, experimental infections would be needed to confirm mechanisms underlying correlations.

We assumed the observed presence-absence  $y(p, i)$  of parasite species  $p$  in host individual  $i$  captured at site  $s$  is a random sample of the population, conditional on host identity, the surrounding environment and individual infection status with other parasites:

$$y(p, i) \sim \text{Bernoulli}[\Psi(p, i)] \quad (\text{eqn. 1})$$

Using a logit-link, we modelled infection probability  $\Psi(p, i)$  of each host individual with parasite  $p$  as:

$$\text{logit}(\Psi(p, i)) \sim \beta_0^P + \beta_{\text{HostSp}}^P(i) + \beta_{\text{Island}}^P(s) + \beta_{\text{Forest}}^P(s) + \gamma_A^P A_{\text{zost.scale}}(s) + E(p, i) \quad (\text{eqn. 2})$$

Here,  $\beta_0^P$  is the parasite-specific intercept, while coefficients  $\beta_{\text{HostSp}}^P$ ,  $\beta_{\text{Island}}^P$ , and  $\beta_{\text{Forest}}^P$  estimate variation in infection probability due to host species, island and forest type, respectively (categorical variables;  $\beta$ -values estimated for each level). Superscript ' $P$ ' is used as coefficients were estimated independently for each parasite species. Coefficient  $\gamma_A^P$  estimates the effect of *Zosterops* proportional abundance  $A_{\text{zost}}$ , estimated as proportion of *Zosterops* individuals from all captured birds at each site. To account for unequal sampling across sites, we modelled  $A_{\text{zost}}$  as a binomial

function of total mistnet captures (all species;  $N_{total}$ ) and *Zosterops* spp. total abundance  $N_{zost}$ :

$$N_{zost}(s) \sim \text{Binomial}(A_{zost}(s), N_{total}(s)) \quad (\text{eqn. 3})$$

Estimates for  $A_{zost}$  were centred and standardised in each iteration ( $A_{zost.scale}$ ).

The term  $E(p,i)$  captures variance-covariance relationships in parasite occurrence in relation to the presence of all parasite species in host individuals (O'Brien & Dunson 2004; Pollock *et al.* 2014). This matrix of random effects is modelled as a zero-centred multivariate normal distribution:

$$E(p,i) \sim \text{MVN}(0, \Omega) \quad (\text{eqn. 4})$$

Here,  $\Omega$  comprises a variance-covariance matrix for which the conjugate prior is a scaled inverse Wishart distribution. The matrix elements describe whether a given parasite pair co-occurs more or less often than expected by chance (based on residual correlations), after accounting for environmental  $\beta^P$  coefficients in eqn. 2. The two parameters of the inverse Wishart are degrees of freedom  $df$  and a positive-definite scale matrix of dimension  $p \times p$  ( $p$  = total number of parasite species). We set  $df = p + 1$  to place a uniform distribution on pairwise correlations, such that values between -1 and 1 were equally likely (Gelman & Hill 2007). To generate correlation estimates, we scaled off-diagonal covariance elements by the diagonals. Standard deviations and correlations in the  $p \times p$  matrix were estimated by multiplying variances of diagonal elements by scaling factors drawn from a *Uniform*(0,100) distribution (Gelman & Hill 2007).

The model was fit in a Bayesian framework with Markov Chain Monte Carlo (MCMC) sampling based on the Gibbs sampler in the freeware JAGS, using the R interface 'rjags' (Plummer 2003). We used normal priors with variance = 2.71 for intercepts and regression coefficients. This prior gives close approximation to a

logistic distribution and is appropriate for estimates on a logit scale when prior information is limited (Lunn *et al.* 2012). To estimate  $A_{zost}(s)$ , we used a  $Beta(2,2)$  distribution truncated between 0.05 and 0.9 (based on observed range limits for  $A_{zost}(s)$ ). For categorical covariates ( $\beta^P_{HostSp}$ ,  $\beta^P_{Island}$ , and  $\beta^P_{Forest}$ ), we used redundancy coefficients to improve convergence and scale estimates (Gelman & Hill 2007). For example, coefficient  $\beta^P_{HostSp}^*$  was calculated for parasite species  $p$  in host species  $h$  as:

$$\beta^P_{HostSp}^*(h) = \beta^P_{HostSp}(h) - \text{mean}(\beta^P_{HostSp})$$

Convergence was assessed visually and posterior predictive checks assessed if model assumptions were good approximations of the data generating process. Bayesian  $p$ -values around 0.5 indicate good fit whereas values near 0 or 1 indicate a discrepancy between predictions and observed data (Gelman, Meng & Stern 1996). While all *Zosterops* individuals were screened for malaria, only 275 birds (from 2014) were screened for microfilaria (note all combinations of host / habitat / island were sampled for microfilaria). Microfilaria data for remaining samples were set as 'NA' (i.e. missing data), allowing the sampler to make inferences from its posterior distribution as if these values were omitted (Lunn *et al.* 2012). This approach ensured inferences were made using the full dataset, rather than excluding individuals or assigning random values, and is appropriate in Bayesian contexts where model-based inference of host-parasite interactions generates less bias than direct data inference (Wells & O'Hara 2013). Where *Haemoproteus* DNA was amplified but no sequence generated and no blood smears existed ( $n = 16$ ), *H. zosteropsis* and *H. killangoi* were also specified as NA.

We ran two chains for 750 000 iterations, discarding 250 000 iterations as burn-in, with a thinning interval of 1000. Results are given as 95% highest posterior

273 credible intervals (CI). We used odds ratios (OR) to compare strength of change in  
274 infection probabilities for levels of categorical covariates. We considered credible  
275 intervals that did not overlap with zero or with those from other covariates as  
276 ‘significant’.

277

278 *Analysis of host heterophil to lymphocyte ratios*

279 We tested for relationships between H/L ratios and infection status for 166 birds from  
280 three *Zosterops* spp. (no infections occurred in *Z. inornatus*; this species was omitted  
281 from H/L analysis) using linear regressions. The response variable was logit-  
282 transformed H/L ratios with assumed normal error distribution. Fixed predictors were  
283 microfilaria, *Haemoproteus*, and *Plasmodium* status (binary variables: infected or  
284 uninfected). Separate models tested each combination of two-way parasite  
285 interactions (triple infections were too rare to test three-way interactions). As time of  
286 day can influence H/L ratios (Banbura *et al.* 2013), we included ‘time’ as a  
287 continuous predictor. We included ‘island’ and ‘host species’ as random grouping  
288 variables, allowing the intercept to vary among groups. A conservative model was  
289 also fit in which *Haemoproteus* and *Plasmodium* infections were combined  
290 (‘malaria’). For model comparisons, we used Akaike’s Information Criterion (AIC),  
291 assuming that a change in AIC of >2 indicates a change in model performance.

292 Data was analysed in R version 3.2.1 (R Core Team, 2008; R: A language and  
293 environment for statistical computing). Data and R code used to perform analyses are  
294 presented in supporting information.

295

296 **Results**

297 *Environmental influences on parasite infection probabilities*

298 In total, 228 of 449 *Zosterops* individuals were infected with haematozoans, including  
299 191 *Haemoproteus*, 88 *Plasmodium* and 41 microfilaria infections (Table 1b; Fig. 1a,  
300 b). Nine avian malaria lineages were morphologically identified to species level for  
301 the first time, including three lineages of *H. killangoi* and four of *H. zosteropsis* (Figs.  
302 S2 - S4). Each of the four focal parasites occurred on all islands, with the exception of  
303 microfilariae (absent from Lifou; Table 1b; Fig. 1b). The multivariate logistic  
304 regression obtained good fit (Bayesian  $p = 0.56$ ). Estimated prevalence across all  
305 individuals ( $\beta_0$ ) was highest for *H. zosteropsis* (CI: 14 - 45%), followed by microfilaria  
306 (5 - 22%), *Plasmodium* spp. (4 - 18%) and *H. killangoi* (2 - 11%).

307 'Forest type' explained 15 - 63% of environmental variation in occurrence  
308 probability for microfilaria, 3 - 65% for *Plasmodium* spp. and 1 - 28% for *H.*  
309 *zosteropsis*, with each parasite less likely to occur in montane rainforest than the two  
310 lowland forest categories (OR: 0.02 - 0.27 for microfilaria, 0.05 - 0.65 for  
311 *Plasmodium* spp. and 0.04 - 0.75 for *H. zosteropsis*). Infection patterns differed across  
312 lowland forest categories, with *H. zosteropsis* and microfilaria more likely to occur in  
313 lowland rainforest (OR: 2.1 - 13.8 and 2.1 - 12.8, respectively) and *Plasmodium* spp.  
314 infections more likely in open lowland forest (OR: 2.1 - 14.5).

315 'Island' explained 7 - 53% of environmental variance in occurrence  
316 probability for microfilaria, 2 - 28% for *H. zosteropsis* and 1 - 68% for *H. killangoi*.  
317 Both *H. zosteropsis* and microfilaria were more likely on Maré than remaining islands  
318 (OR: 3.7 - 37.3 and 1.9 - 13.1, respectively; Fig. 1c). Infections with *H. killangoi* were  
319 more likely on Ouvéa (OR: 1.1 - 12.1; Fig. 1c). In addition to island and habitat  
320 effects, *H. zosteropsis* occurrence was negatively influenced by *Zosterops* spp.  
321 'proportional abundance' [explaining 6 - 91% of variation in infection probability

(OR: 0.01 - 0.69)]. Variance explained by ‘host species’ overlapped with zero for all parasites and credible intervals overlapped among different host species.

*Co-infections and parasite co-occurrence probabilities*

A total of 82 parasite co-infections were observed, accounting for 35.9% of all infected birds and representing all pairwise parasite combinations (Table 1c). We observed 13 *H. zosteropsis*/*Plasmodium*/Microfilaria triple infections and one *H. killangoi*/*H. zosteropsis*/*Plasmodium* triple infection. After accounting for environmental covariates, estimated covariances revealed ‘significantly’ correlated infection probabilities for all parasite pairs apart from *H. zosteropsis* / *Plasmodium* spp. (Fig. 2). Infection probabilities for two of three pairwise avian malaria combinations were negatively correlated, with the third showing a non-significant negative trend (Fig. 2). All observed microfilariae co-occurred with malaria (Table 1), and microfilaria infections correlated positively with occurrences of *Plasmodium* spp. and *H. zosteropsis*, but negatively with *H. killangoi* (Fig. 2). In fact, thirty-three of 44 observed microfilaria infections co-occurred with *H. zosteropsis*, while co-infections of any parasite with *H. killangoi* were rare (accounting for five of 52 observed *H. killangoi* infections; Table 1c).

*Relationship between parasite infections and host heterophil to lymphocyte ratios*

Microfilariae were associated with increased H/L ratios when accounting for time and presence of other parasites ( $\Delta AIC$  without microfilaria: +11.17; Fig. 3). This elevation was driven by increased heterophils (mean with microfilaria:  $12.73 \pm 2.21$ ; without:  $5.03 \pm 0.45$ ) and decreased lymphocytes (mean with microfilaria:  $74.93 \pm 2.28$ ; without:  $82.68 \pm 0.85$ ). Neither *Haemoproteus* nor *Plasmodium* spp. influenced



347 H/L ratios, either as separate variables or combined ( $\Delta$ AIC without *Haemoproteus*: -  
348 2.91; without *Plasmodium*: -2.82; without 'malaria': -1.11; Fig. 3).

349

## 350 Discussion

351 We provide a rare demonstration of apparent biotic associations between wildlife  
352 parasites. Two widespread *Haemoproteus* parasites had dissimilar co-infection  
353 patterns and a negative co-occurrence probability, a pattern indicative of competition  
354 between parasites that utilise the same host resources. Birds with microfilariae had  
355 elevated H/L ratios and two avian malaria parasites (*H. zosteropsis* and *Plasmodium*  
356 spp.) had positive co-occurrence probabilities with microfilaria, consistent with  
357 evidence that nematode-induced immune modulation may facilitate malaria co-  
358 infections (Druilhe, Tall & Sokhna 2005). Our results indicate that interspecific  
359 associations are an important but overlooked mechanism influencing wildlife parasite  
360 infections.

361

### 362 *Correlated infection probabilities: evidence of parasite competition and facilitation?*

363 We identified negative parasite co-occurrence probabilities between *H. zosteropsis* / *H.*  
364 *killangoi* and between *H. killangoi* / *Plasmodium* spp., supporting our prediction that  
365 interspecific malaria infections would be negatively correlated. Only two co-  
366 infections were observed for each of the above parasite pairs, despite each parasite  
367 occurring on all islands and habitats. Considering that *H. zosteropsis* and *H. killangoi*  
368 are avian host specialists that appear restricted to Zosteropidae (Valkiūnas 2005;  
369 Clark & Clegg 2015), our results may be evidence of interspecific competition. We  
370 also found a striking difference in likelihoods of microfilaria co-infection for the two  
371 *Haemoproteus* species. We predicted malaria infections would positively correlate

372 with microfilaria; yet, while no filarial parasites occurred in birds free from avian  
373 malaria, birds carrying *H. killangoi* rarely carried microfilaria. In comparison, birds  
374 carrying *H. zosteropsis* had increased likelihood of carrying microfilaria when  
375 accounting for their similar environmental affiliations. Contrasting patterns for host-  
376 specialist *Haemoproteus* parasites suggest associations with immune-modulating  
377 nematodes are uneven between rival malaria species, a fascinating finding that  
378 deserves further attention in field and laboratory studies.

379       Explaining patterns of co-occurrence for vector-borne parasites requires  
380 careful consideration of the role of vectors. Similarly to previous studies, we found  
381 important environmental influences on blood parasite distributions (Lachish *et al.*  
382 2011; Oakgrove *et al.* 2014; Sehgal 2015). Despite wide CIs owing to uncertainty, we  
383 identified habitat and island infection patterns that likely reflect distributions of  
384 arthropod vectors (Rogers *et al.* 2002; Santiago-Alarcon, Palinauskas & Schaefer  
385 2012). Both *Haemoproteus* and microfilaria are known to use Ceratopogonid midges  
386 as vectors, and evidence suggests that different *Haemoproteus* parasites can use  
387 different Ceratopogonid species (Santiago-Alarcon, Palinauskas & Schaefer 2012).  
388 Associations between *H. zosteropsis* and microfilaria could be evidence of a shared  
389 vector, while a different vector may transmit *H. killangoi*, perhaps reducing co-  
390 infections. This hypothesis adds to the growing need for future studies of  
391 haematozoan vectors (Clark, Clegg & Lima 2014; Bobeva *et al.* 2015; Žiegytė &  
392 Valkiūnas 2015; Bernotienė & Valkiūnas 2016). In addition to environmental effects,  
393 a surprising finding was the negative influence of *Zosterops* spp. proportional  
394 abundance on *H. zosteropsis* occurrence. The idea that hosts reach higher abundance  
395 where infections are lower touches on exciting evolutionary questions, such as host-

396 parasite interactions driving taxon cycles (Ricklefs *et al.* 2016) or shaping host  
397 dispersal patterns (Poulin *et al.* 2012; Aharon-Rotman *et al.* 2016).

398       Our data was not complete, as only samples from 2014 were subject to smear  
399 and genus-specific PCR screening, adding to uncertainty in our estimates and  
400 emphasising the need for greater scrutiny of co-occurring wildlife pathogens (Petney  
401 & Andrews 1998; Knowles 2011). In addition to incomplete data, some parasite  
402 associations seen here could have been inflated by missing covariates (Pollock *et al.*  
403 2014), as we lacked microhabitat data such as temperature and moisture that can  
404 influence local transmission (Zamora-Vilchis, Williams & Johnson 2012; Cornuault  
405 *et al.* 2013; Sehgal 2015). Due to complex environmental influences and the inherent  
406 uncertainty in pathogen observations, we propose that multivariate logistic regression  
407 combined with appropriate covariate data provides a useful platform for analyses of  
408 wildlife pathogen associations.

409

#### 410 *Altered heterophil to lymphocyte ratios in malaria/microfilaria coinfections*

411 Though often overlooked, haematozoan co-infections are important, as they may  
412 compound effects on host condition and survival (Valkiūnas *et al.* 2006; Palinauskas  
413 *et al.* 2011; Oakgrove *et al.* 2014; Dimitrov *et al.* 2015). Yet identifying mechanisms  
414 that drive wildlife parasite associations is challenging (Cattadori, Boag & Hudson  
415 2008; Tompkins *et al.* 2011). Our finding of altered H/L ratios during microfilaria  
416 infection identifies immune modulation as a possible mechanism by which parasitic  
417 nematodes may facilitate co-occurring malaria. Microfilariae led to decreased  
418 lymphocytes and increased heterophils, changes that could decrease a host's ability to  
419 regulate pathogens through antigen recognition (Pedersen & Fenton 2007; Bordes &  
420 Morand 2011). We did not observe changes in H/L ratios in birds carrying malaria but

421 not microfilaria, consistent with prior studies (Ricklefs & Sheldon 2007) and  
422 suggesting the presence of parasitic nematodes drove these changes. This pattern  
423 supports laboratory evidence that microfilariae depress adaptive immune pathways  
424 responsible for identifying infections while increasing neutrophil-associated  
425 inflammation (Druilhe, Tall & Sokhna 2005).

426       Increases in disease have been observed for many pathogens that co-occur  
427 with nematodes, including HIV in humans (Bentwich *et al.* 1999). However this  
428 relationship is not always facilitatory, as some nematodes depress co-occurring  
429 malaria by reducing target cell densities (Griffiths *et al.* 2015). While positive  
430 correlations between *H. zosteropsis* and microfilaria may indicate interspecific  
431 facilitation, we stress that experimental perturbations and assessment of host  
432 immunity are necessary to clarify within-host interactions (Sheldon & Verhulst 1996;  
433 Johnson & Buller 2011; Knowles *et al.* 2013). In addition, data that takes into account  
434 changes in parasite density during co-infection could provide clues as to how  
435 coinfections alter disease progression (Metcalf *et al.* 2016). Although we cannot  
436 speculate on within-host dynamics, our results contribute to a growing recognition  
437 that parasitic nematodes are important components of pathogen epidemiology (Petney  
438 & Andrews 1998; Nacher *et al.* 2001).

439

#### 440 *Conclusions*

441 We present evidence that biotic associations play important roles in the occurrences  
442 and infection likelihoods of haematozoan parasites. Our description of parasite co-  
443 occurrence patterns provides critical new insights into disease ecology, as parasite  
444 associations are expected across many host systems (Bell *et al.* 2006; Pérez-Tris *et al.*  
445 2007; Johnson & Buller 2011; Vaumourin *et al.* 2015), yet evidence from wildlife is

446 biased towards mammalian hosts (Lello *et al.* 2004; Tompkins *et al.* 2011; Hellard *et*  
447 *al.* 2015). Additionally, we show that co-infections are difficult to identify using PCR  
448 alone, a finding demonstrated for many host-pathogen systems (Valkiūnas *et al.* 2006;  
449 Dyachenko *et al.* 2010; Grybchuk-Ieremenko *et al.* 2014; Moustafa *et al.* 2016). We  
450 overcame this hurdle by combining traditional and molecular parasitology methods, a  
451 multidisciplinary approach that we recommend for future work on wildlife co-  
452 infections.

453

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465

#### 466 **Data accessibility**

467 Malaria lineages will be deposited in GenBank and the MalAvi database. Microfilaria  
468 LSU lineages will be deposited in GenBank. Data and R code used for analyses will  
469 be deposited to Dryad upon acceptance.

470

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- 747

748 Table 1: (a) *Zosterops* spp. sample sizes across New Caledonian islands (numbers in  
 749 italics indicate published samples) included in the multivariate logistic regression. (b)  
 750 observed haematozoan parasite infections and (c) co-infections across islands. Note  
 751 that 449 samples were screened for *Haemoproteus* and *Plasmodium* spp., while 275  
 752 samples were screened for microfilariae.  
 753

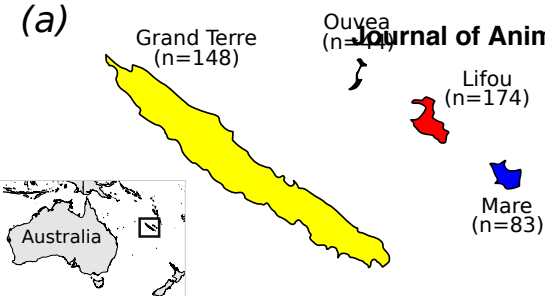
|                                   |                        |              |                     |         |
|-----------------------------------|------------------------|--------------|---------------------|---------|
| (a) <i>Zosterops</i> host species | Grand Terre            | Maré         | Ouvéa               | Lifou   |
| <i>Z. lateralis</i>               | 10 (26)                | 5 (20)       | 44 (0)              | 27 (20) |
| <i>Z. xanthochrous</i>            | 69 (43)                | 38 (20)      | absent              | absent  |
| <i>Z. minutus</i>                 | absent                 | absent       | absent              | 72 (25) |
| <i>Z. inornatus</i>               | absent                 | absent       | absent              | 10 (20) |
|                                   |                        |              |                     |         |
| (b) Haematozoan parasites         |                        |              |                     |         |
| <i>Haemoproteus zosteropis</i>    | 60                     | 36           | 14                  | 9       |
| <i>H. killangoi</i>               | 28                     | 5            | 11                  | 8       |
| <i>Plasmodium</i> spp.            | 76                     | 7            | 3                   | 2       |
| Microfilaria                      | 25                     | 12           | 7                   | 0       |
|                                   |                        |              |                     |         |
| (c) Observed co-infections        | <i>Plasmodium</i> spp. | Microfilaria | <i>H. killangoi</i> |         |
| <i>H. zosteropis</i>              | 28                     | 33           | 2                   |         |
| <i>H. killangoi</i>               | 2                      | 1            | -                   |         |

754  
 755

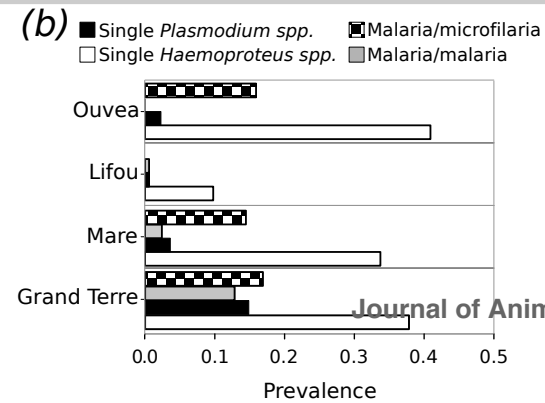
756 **Figure legends**

757  
758 Fig. 1: (a) *Zosterops* spp. sample sizes (n) on New Caledonian islands. (b)  
759 Observations of haematozoan parasite infections and co-infections. Note that only 275  
760 samples were screened for microfilaria. (c) Estimated odds ratios of infection  
761 probability across islands. Presented are posterior modes, 50% highest posterior  
762 density credible intervals (thick lines) and 95% highest posterior density credible  
763 intervals (thin lines). Colours of symbols correspond to colours of islands in (a). (Full  
764 page figure, 182mm)  
765  
766 Fig. 2: Haematozoan parasite pairwise correlations of infection probabilities.  
767 Correlations were estimated from a parasite variance-covariance matrix after  
768 accounting for environmental covariates in a multivariate logistic regression. Shading  
769 indicates 95% highest posterior density credible intervals. *Plas.*, *Plasmodium* spp.;  
770 *Mf.*, microfilaria (Single column figure, 70mm)  
771  
772 Fig. 3: Heterophil to lymphocyte ratios for *Zosterops* spp. across parasite infection  
773 classes. Also presented are total sample sizes (n) for each infection. MF, microfilaria  
774 (Single column figure, 70mm)  
775

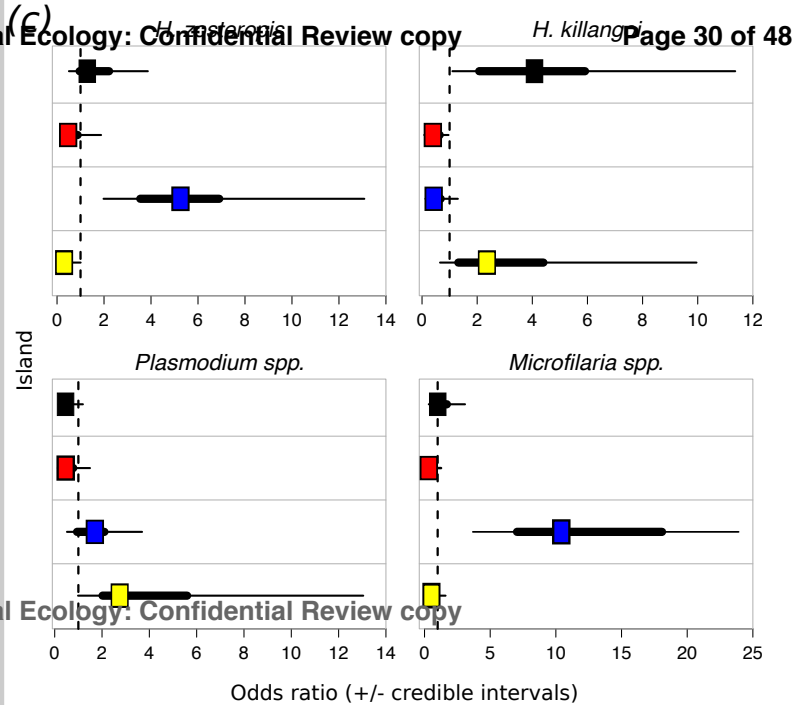
(a)



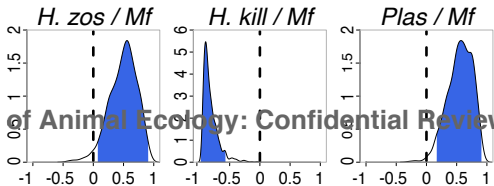
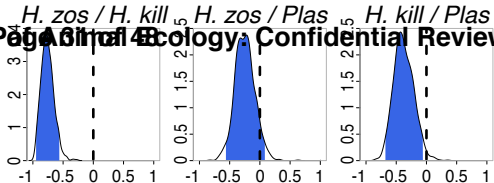
(b)



(c)



Posterior density

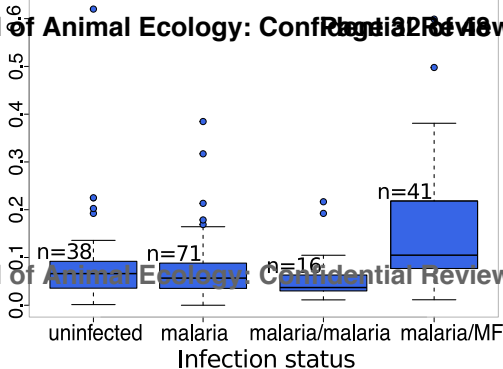


Covariance correlation



H/L ratio

Journal of Animal Ecology: Confidential Review



Clark et al. JAE Supporting Information

### Distribution of sample sites across habitats and islands in New Caledonia

Sample sites were chosen to represent the three primary forested habitats that occur in New Caledonia. These include montane rainforest (occurring in upper elevations along the central midline of Grand Terre), dry lowland forest [which occur in on the western (leeward) side of Grand Terre and on the north-western end of Ouvéa] and lowland rainforest (the principle habitat on the outer islands of Ouvéa, Lifou and Maré; Fig. S1). The final dataset included *Zosterops* infection data and *Zosterops* relative abundance data from 17 sites (Fig. S1).

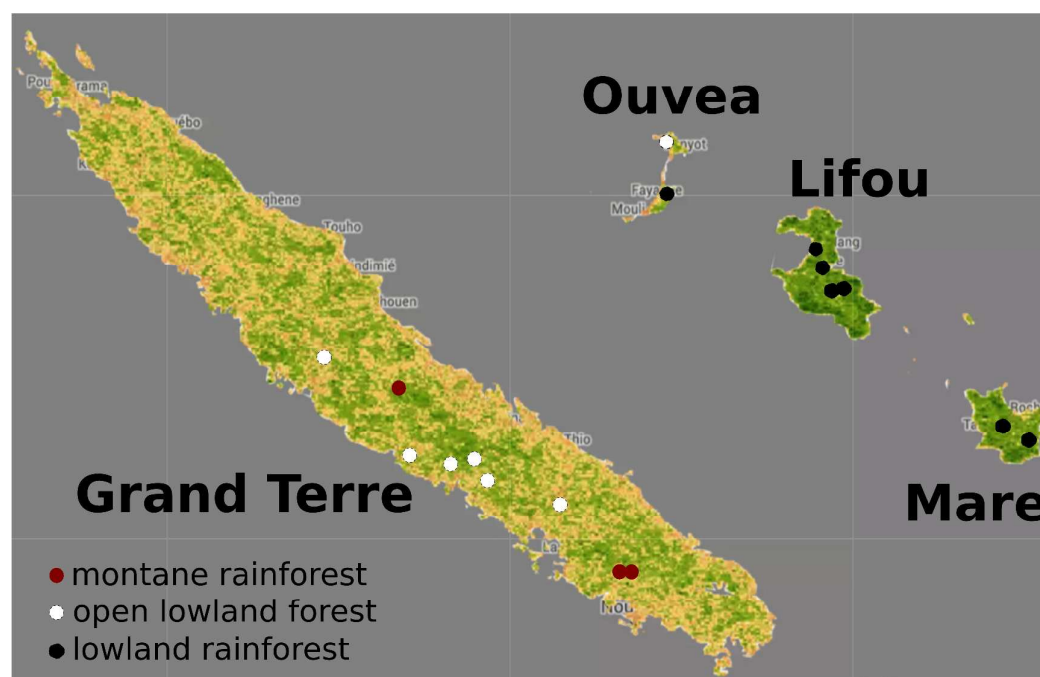


Fig. S1: Distribution of sample sites across islands and forested habitat types in New Caledonia. Colours of island landscapes represent a heat map of habitat heterogeneity, with warmer colours indicating higher heterogeneity (map accessed at <http://www.earthenv.org>)

### Phylogenetic reconstruction and species identification of avian malaria lineages

#### Methods

We constructed a molecular phylogeny to estimate avian malaria lineage relationships. We used Akaike's information criterion in jModelTest (v 0.1.1; Posada & Crandall 1998) to determine the evolutionary model (GTR+I+G) and we used BEAST for phylogenetic reconstruction (v 1.8.1; Drummond & Rambaut 2007). We used a Yule prior for branching rates. We carried out two runs of 20 million generations, sampling at every 1000 generations for each run. We used TRACER (v 1.5; Rambaut & Drummond 2007) to test if estimated sample size (ESS) for each parameter was sufficient ( $ESS > 200$ ) for robust estimates. We

Clark et al. JAE Supporting Information

discarded four million generations of burn-in per run, leaving a distribution of 32000 trees. Three mammalian *Plasmodium* *cyt-b* sequences were used as outgroups. To facilitate reconstruction of relationships between morphospecies, we included malaria lineages previously detected in Australian and New Caledonian *Zosterops* spp. (Ishtiaq *et al.* 2010; Clark & Clegg 2015).

### Results

We recorded 14 avian malaria lineages (six *Haemoproteus* and eight *Plasmodium*), including seven new lineages (GenBank Accessions: XXXX). Phylogenetic reconstruction revealed two distinct *Haemoproteus* clades. For Clade I, smears from four of the six lineages were inspected, with all four confirmed as the morphospecies *H. zosteropsis*. We therefore assumed that all lineages in this clade represent *H. zosteropsis* (mean within-clade divergence=1.6%; Fig. S1). The second *Haemoproteus* clade was separated from the *H. zosteropsis* clade by a mean divergence of 6.2% and was identified as *H. killangoi* (visual confirmation for three of the four lineages; Fig. S1). Both morphospecies were present on all islands.

*Plasmodium* lineages grouped into four clades, two of which formed the majority of *Plasmodium* lineages (12 of 16 lineages; figure S2). One of the main *Plasmodium* clades contained lineage GRW06, previously identified as *P. elongatum* by Valkiūnas *et al.* (2008). Visual inspection of smears for another lineage within this clade also identified *P. elongatum* (MYNA01P). The second major *Plasmodium* clade included lineage GRW4, previously confirmed as *P. relictum* by Beadell *et al.* (2009), and a second lineage that we confirmed as *P. relictum* (NC8P). We therefore refer to these clades as *P. elongatum* (mean within-clade divergence=2.1%) and *P. relictum* (mean within-clade divergence=1.9%).

We sequenced 41 microfilaria infections and identified three new LSU lineages (GenBank Accession numbers XXXX). Two LSU lineages occurred across the entire microfilaria distribution (ZOSMF1, ZOSMF3). The third lineage (ZOSMF2) was rare, with two recordings on Grand Terre. ZOSMF1 and ZOSMF2 were separated by a single base pair (0.1% nucleotide divergence), while ZOSMF3 was more distinct (6.0% divergence from ZOSMF1).

Sample sizes were too sparse to include each *Plasmodium* morphospecies separately in the multivariate logistic regression (MCMC chains did not reach convergence), and so *Plasmodium* infections were grouped together in a single variable. Our regression model therefore included infection data from four parasite groups (*H. zosteropsis*, *H. killangoi*, *Plasmodium* spp. and microfilaria spp.)

Clark et al. JAE Supporting Information

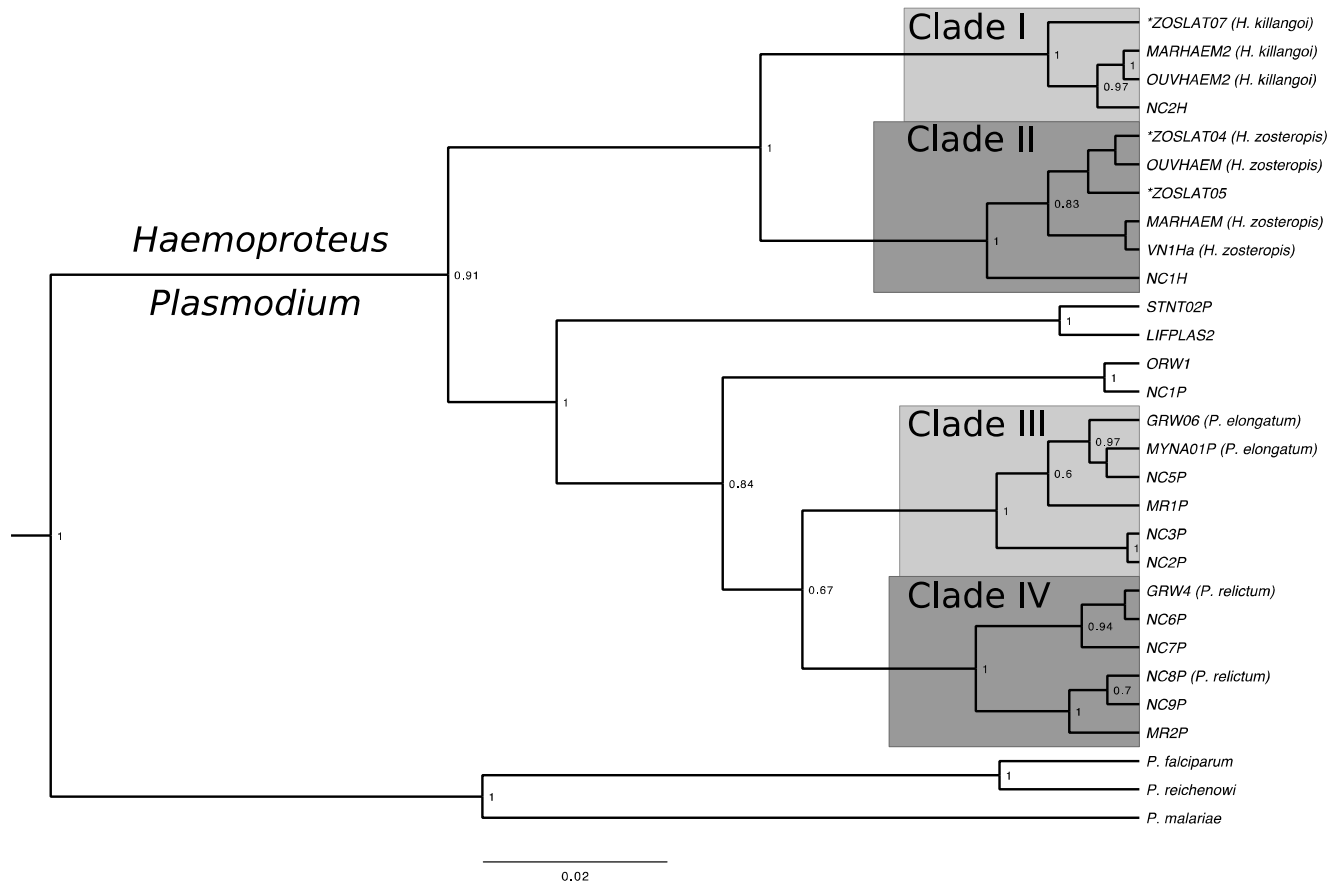


Fig. S2: Bayesian molecular phylogeny of avian malaria cyt-*b* lineages recorded in New Caledonian *Zosterops* spp. Lineages that were previously recorded in New Caledonian and Australian *Zosterops* spp. were included to improve phylogenetic reconstruction (Ishtiaq et al., 2010; Olsson-Pons et al., 2015). Lineages with asterisks (\*) were recorded in Australian *Zosterops* spp. Shaded regions represent monophyletic clades that were visually associated with known parasite morphospecies (Clade I, *Haemoproteus killangoi*; Clade II, *H. zosteropsis*; Clade III, *Plasmodium elongatum*; Clade IV, *P. relictum*). Numbers at nodes represent Bayesian posterior probabilities of branch placement.

Clark et al. JAE Supporting Information

### Parasite morphological descriptions and DNA barcoding

*Haemoproteus (Parahaemoproteus) zosteropis* Chakravarty and Kar, 1945

DNA sequences: Mitochondrial *cyt b* lineages ZOSLAT04, MARHAEM, OUVHAEM and VN1H (479 bp, GenBank accession numbers XXXXX).

Avian host and distribution: The lineage ZOSLAT04 has been recorded in the silvereye *Zosterops lateralis* in eastern Australia (Zamora-Vilchis, Williams & Johnson 2012; Clark & Clegg 2015; Clark *et al.* 2015). Lineage OUVHAEM was recorded in *Z. lateralis* on the island of Ouvea, New Caledonia. Lineage MARHAEM was recorded in *Z. lateralis* and the green-backed white-eye *Z. xanthochrous* on the island of Mare, New Caledonia. Lineage VN1H has been recorded in various species of white-eye throughout New Caledonia and Vanuatu (Olsson-Pons *et al.* 2015). All infections were recorded in birds caught in forested habitats using mistnets.

Site of infection: Mature erythrocytes; no other data.

Representative blood films: Voucher specimens (accession numbers XXXX) were deposited in the Queensland Museum, Brisbane, Australia. Labels data for voucher slides are: XXXX, collected by N. Clark and S. Clegg.

**Young gametocytes** (Fig. S3a, b) were occasionally seen in the voucher preparations, usually situated at the distal end of the infected erythrocyte; the growing gametocytes not seen in the voucher; the outline even.

**Macrogametocytes** (Fig. S3c–h) grow along erythrocyte nuclei and slightly enclose them with ends. Growing gametocyte usually adheres to the erythrocyte envelope and do not touch the erythrocyte nucleus forming cleft-like space between gametocyte and erythrocyte nucleus (Fig. S3c, d). Ends of medium grown gametocytes rounded (Fig. S3e). Fully-grown gametocyte filling erythrocyte up to the poles, closely appressed to the erythrocyte envelope and erythrocyte nucleus, not displace or slightly displaces the nucleus laterally (Fig. S3g, h). Dumbbell-shaped forms absent. Mature gametocytes only slightly enclose erythrocyte nuclei and never encircle them (Fig.

Clark et al. JAE Supporting Information

S3h). The cytoplasm finely granular in appearance, frequently contains several small vacuoles usually situated next to pigment granules (Fig. S3c, g, h). The outline even. The parasite nucleus variable in shape, often roundish or oval, usually subterminal in position (Fig. S3f, g). Pigment granules are oval and roundish, of medium (0.5 to 1.0  $\mu\text{m}$ ) size, randomly scattered throughout the cytoplasm; their number is between 12 and 17 (on average  $15.1 \pm 1.0$ ).

**Microgametocytes** (Fig. S3i–l). General configuration and other features are as for macrogametocytes, with usual haemosporidian sexual dimorphic characters (Valkiūnas 2005). Pigment granules roundish or rod-like of medium (0.5 to 1.0  $\mu\text{m}$ ) and big (1.0 to 1.5  $\mu\text{m}$ ) size; their number 11–17 (on average  $13.8 \pm 2.0$ ).

Remarks: *Haemoproteus zosteropsis* has been frequently recorded in co-infection with *Haemoproteus killangoi* (primarily in Africa; Valkiūnas 2005), though co-infections in New Caledonia appear to be rare. *Haemoproteus zosteropsis* can be easily distinguished from *H. killangoi* based on the even outline and lack of dumbbell-shaped gametocytes. Note that the parasites from our material differ from the original neohapantotype of *H. zosteropsis* (see Valkiūnas 2005, pp. 391–394) by prominent cleft-like space between of growing macrogametocytes and erythrocyte nucleus.

*Haemoproteus (Parahaemoproteus) killangoi* Bennett and Peirce, 1981

DNA sequences: Mitochondrial cyt *b* lineages ZOSLAT07, OUVHAEM2, MARHAEM2 (479 bp, GenBank accession number XXXXX).

Avian host and distribution: The lineage ZOSLAT07 was recorded in the silvereye *Zosterops lateralis* in southeast Queensland, Australia. Lineage OUVHAEM2 was recorded in *Z. lateralis* on the island of Ouvea, New Caledonia. Lineage MARHAEM2 was recorded in the green-backed white-eye *Z. xanthochrous* on the island of Mare, New Caledonia. All infections were recorded in birds caught in forested habitats using mistnets.

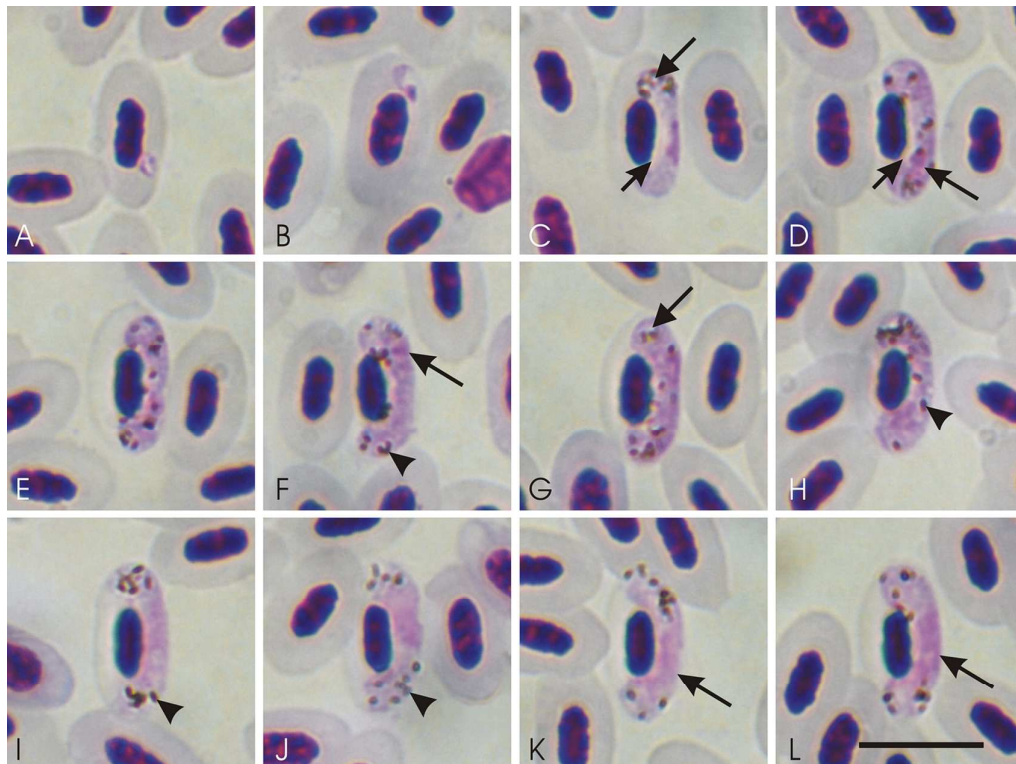


Fig. S3: Young gametocytes (a, b), macrogametocytes (c–h) and microgametocytes (i–l) of *Haemoproteus zosteropsis* from the blood of the silvereye *Zosterops lateralis*. Giemsa-stained thin blood films. Long simple arrows—nuclei of parasites; short simple arrows—unfilled spaces between gametocytes and erythrocyte nucleus; long triangle arrows—vacuoles; simple arrowheads—pigment granules. Scale bar=10  $\mu$ m

Site of infection: Mature erythrocytes; no other data.

Representative blood films: Voucher specimens (accession numbers XXXX) were deposited in the Queensland Museum, Brisbane, Australia. Labels for deposited slides are: XXXX, collected by N. Clark and S. Clegg.

**Young gametocytes** were not seen in voucher preparations.

**Macrogametocytes** (Fig. S4a–d) grow around erythrocyte nuclei and enclose them with ends but not encircle them completely. Fully grown gametocytes displace erythrocyte nucleus laterally (Fig. S4d). Enucleated erythrocytes absent. Cytoplasm finally granular, stains dark blue (basophilic) with Giemsa. Outline highly amoeboid in growing (Fig. S4a–b) and even in the fully grown gametocytes (Fig. S4d).

Advanced gametocytes adhere to nuclei fill erythrocytes up to the poles, but not

Clark et al. JAE Supporting Information

touching the central part of erythrocytes envelope causing “dip” giving dumbbell-like appearance (Fig. S4a–c). Dumbbell-shaped forms of advanced gametocytes predominate in the vouchers (80 %), but fully grown gametocyte lose dumbbell-like shape (Fig. S4d). Parasite nucleus compact, variable in shape, subterminal in position (Fig. S4b, c). Pigment granules roundish of medium size (0.5 to 1.0  $\mu\text{m}$ ) (Fig. S4a) and rod-like of large size (1.0 to 1.5  $\mu\text{m}$ ) (Fig. S4b–d); their number 7–13 (on average  $10.1 \pm 1.7$ ).

**Microgametocytes** (Fig. S4e–h). General configuration and other features are as for macrogametocytes, with usual haemosporidian sexual dimorphic characters (Valkiūnas 2005). Pigment granules roundish of medium size (0.5 to 1.0  $\mu\text{m}$ ) and rod-like of large size (1.0 to 1.5  $\mu\text{m}$ ); their number 6–12 (on average  $9.4 \pm 1.4$ ).

Remarks: *Haemoproteus killangoi* can be readily distinguished from *H. zosteropsis* based on amoeboid outlines, dumbbell shaped forms of the growing gametocytes, less number of the pigment granules and basophilic stain of the cytoplasm.

Macrogametocytes of *H. killangoi* are predominantly nucleophilic in contrast with those of *H. zosteropsis*, which closely appressed to the envelope of the erythrocyte and form prominent cleft-like space between of growing macrogametocytes and erythrocyte nucleus.

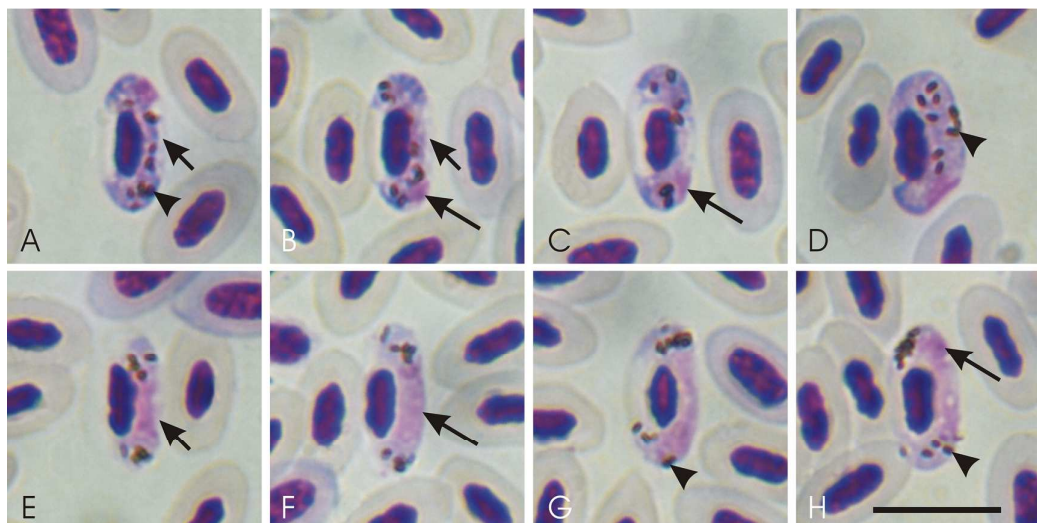


Fig. S4: Macrogametocytes (a–d) and microgametocytes (e–h) of *Haemoproteus killangoi* from the blood of the green-backed white-eye *Zosterops xanthochrous*. Giemsa-stained thin blood films. Long simple arrows—nuclei of parasites; short simple arrows—unfilled spaces between gametocytes and erythrocyte envelope; simple arrowheads—pigment granules. Scale bar=10  $\mu\text{m}$



Clark et al. JAE Supporting Information

Table S1: Parasite PCR primers and reaction conditions. TD = touchdown (drop the anneal temperature by 1 degree per cycle)

|   | Primers  |          | Volumes               |               |                                 | Cycling Conditions  |                              |                | Source                                      |
|---|----------|----------|-----------------------|---------------|---------------------------------|---|------------------------------|----------------|---|
|   | Forward  | Reverse  | Qiagen TopTaq PCR mix | Primer (each) | DNA (extraction or PCR product) | Cycle times   | Annealing temps and # cycles |                |   |
| <b><i>Malaria</i></b>                       |          |          |                       |               |                                 |   |                              |                |   |
| Nested PCR (Outside Rxn)                    | HAEMNF   | HAEMNR 2 | 10uL                  | 0.5uL         | 3uL (Extraction)                | Denature (30 sec),<br>Anneal (35 sec),<br>Extend (45 sec) | 60 to 55 TD (6 cycles)       | 53 (20 cycles) | Waldenström et al. 2004                     |
| Nested PCR (Inside Rxn)                     | HAEMF    | HAEMR2   | 10uL                  | 0.5uL         | 0.8uL (Nested Outside Product)  | Denature (30 sec),<br>Anneal (35 sec),<br>Extend (45 sec) | 60 to 55 TD (6 cycles)       | 51 (30 cycles) | Waldenström et al. 2004                     |
| <i>Haemoproteus</i> -specific Amplification | HMONLY F | HMONLY R | 10uL                  | 0.5uL         | 3uL (Extraction)                | Denature (30 sec),<br>Anneal (35 sec),<br>Extend (45 sec) | 60 to 55 TD (6 cycles)       | 51 (30 cycles) | This study                                  |
| <b><i>Microfilaria</i></b>                  |          |          |                       |               |                                 |   |                              |                |   |
| LSU Screening and Amplification             | Nem 1    | Nem 2    | 10uL                  | 0.5uL         | 3uL (Extraction)                | Denature (30 sec),<br>Anneal (40 sec),<br>Extend (45 sec) | 60 to 56 TD (5 cycles)       | 55 (30 cycles) | Nadler & Hudspeth, 1998; Sehgal et al. 2005 |
| <b><i>New Primer sequences</i></b>          |          |          |                       |               |                                 |   |                              |                |   |
| HMONLY-F: GCAYGCTACTGGTGCTACAT              |          |          |                       |               |                                 |   |                              |                |   |
| HMONLY-R: TGCATTATCAGGATGAGCTARTGG          |          |          |                       |               |                                 |   |                              |                |   |

Clark et al. JAE Supporting Information

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```

##Avian haematozoan parasite co-occurrences and H/L ratios analyses
library(lme4)
library(rjags)
library(MCMCpack)
library(runjags)

## Read data, set working directory
Dat <- read.csv("Clark_et_al_Supplement_RawData.csv", header=TRUE, stringsAsFactors = FALSE)

## Convert capture sessions to numeric variable
Dat$num.capture.session <- as.numeric(as.factor(Dat$Capture.session))

# Subset Zosterops data for simpler calculations of sample size and observed parasite occurrences
ZosDat<-Dat[ which(Dat$Genus=="Zosterops"),]
.d_indID <- ZosDat$Bird
.d_bird.species <- ZosDat$Species
.d_island <- ZosDat$Island
.d_capture.session <- ZosDat$num.capture.session
.d_genus <- ZosDat$Genus
.d_habitat <- ZosDat$Habitat
.d_Infected<-ZosDat$Infected
.d_infect.H.zosteropis <- ZosDat$H.zosteropis
.d_infect.H.killangoi <- ZosDat$H.killangoi
.d_Haem<-ZosDat$Haem
.d_Plas<-ZosDat$Plas
.d_infect.Microfilaria <- ZosDat$Microfilaria
.d_HLratio <- ZosDat$H.L.Ratio
.d_time <- ZosDat$Time

#####
# Model co-occurrence patterns of parasites
#####

# Data to BUGS/JAGS
island <- as.numeric(as.factor(.d_island))
habitat <- as.numeric(as.factor(.d_habitat))
hostspec <- as.numeric(as.factor(.d_bird.species))
capture.session <- as.numeric(as.factor(.d_capture.session))
nhost <- length(unique(.d_bird.species))
nisland <- length(unique(.d_island))
nhabitat <- length(unique(.d_habitat))
nind <- length(.d_indID )
ncapture.session <- length(unique(.d_capture.session))

# Calculate total number of Zosterops captured at each site using full dataset "Dat"
nzos <- rep(NA, ncapture.session)
for(i in 1:ncapture.session){
  nzos[i] <- as.numeric(length(which(Dat$num.capture.session==i & Dat$Genus=="Zosterops")))}

#Calculate total number of all birds captured at each site
ntotal <- rep(NA, ncapture.session)
for(i in 1:ncapture.session){
  ntotal[i] <- as.numeric(length(which(Dat$num.capture.session==i)))}

## Number of parasite species in modelled data
nparasite <- 4

```

```

# Identity matrix for inverse Wishart prior
W.id <- diag(nparasite)

# Create array of parasite occurrence data
InfestData <- array(NA, dim = c(nind, nparasite))
InfestData[,1] <- .d_infect.H.zosteropis
InfestData[,2] <- .d_infect.H.killangoi
InfestData[,3] <- .d_Plas
InfestData[,4] <- .d_infect.Microfilaria

data2bugs_malaria <- list(
  InfestData = InfestData,
  nhost = nhost,
  nind = nind,
  nhabitat = nhabitat,
  nparasite = nparasite,
  nisland = nisland,
  ncapture.session = ncapture.session,
  nzos = nzos,
  ntotal = ntotal,
  island = island,
  habitat = habitat,
  hostspec = hostspec,
  capture.session = capture.session,
  W.id = W.id
)

#####
# BUGS/JAGS model for multi-species parasite occurrence probabilities
#####
cat(
"model {
  for (i in 1:nind){
    for (par in 1:nparasite){

## Likelihood: link data to model
InfestData[i, par] ~ dbern(p[i, par])

## Logit link function
p[i, par] <- exp(Z[i, par]) / ( exp(Z[i, par]) + 1)

## Model of environmental covariates
Mu[i, par] <- mu0[par] + b.hostsp.star[par, hostspec[i]] + b.island.star[par, island[i]]
+ b.habitat.star[par, habitat[i]] + beta1[par] * prop.zosterops.scale[capture.session[i]]
}

## Multivariate model of interspecific parasite co-occurrence in any bird
Z[i, 1:nparasite] ~ dmnorm(Mu[i, ], Tau[,])
}

## Priors.
##Normal w/ variance 2.71 (dnorm(x,0.368), appropriate for logit estimates (Lunn et al. 2012)
## mu0 mean -1 centres prev. estimates ~ 27% (inv.logit(-1)=0.27), more realistic than mean 0 (50% prev)
for(par in 1:nparasite) {
  mu0[par] ~ dnorm(-1, 0.368)
  beta1[par] ~ dnorm(0, 0.368)
}

```

```

for(h in 1:nhost){
  b.hostsp[par, h] ~ dnorm(0, 0.368)
##Redundancy parameters for covariates (.star) help speed up convergence
  b.hostsp.star[par, h] <- b.hostsp[par, h] - mean(b.hostsp[par,])
}

for(b in 1:nisland){
  b.island[par, b] ~ dnorm(0, 0.368)
  b.island.star[par, b] <- b.island[par, b] - mean(b.island[par,])
}

for(d in 1:nhabitat){
  b.habitat[par, d] ~ dnorm(0, 0.368)
  b.habitat.star[par, d] <- b.habitat[par, d] - mean(b.habitat[par,])
}
}

## Model proportion of Zosterops in each site, with possible values between 0.05 and 0.9
## Centre and standardise prop.zosterops estimates to improve interpretation of model intercept mu0
for(c in 1:ncapture.session){
  nzos[c] ~ dbinom(prop.zosterops[c], ntotal[c])
  prop.zosterops[c] ~ dbeta(2,2)T(0.05,0.9)
  prop.zosterops.scale[c] <- (prop.zosterops[c] - prop.zosterops.mean) / prop.zosterops.sd
}
prop.zosterops.mean <- mean(prop.zosterops[])
prop.zosterops.sd <- sd(prop.zosterops[])

#Scaled inverse Wishart, (equally likely with values between -1 and 1; Gelman & Hill 2007)
#df = K+ 1 sets uniform distribution on individual correlation parameters
Tau[1:nparasite, 1:nparasite] ~ dwish(W.id[, ], df)
df <- nparasite + 1
Sigma.Covar.raw[1:nparasite, 1:nparasite] <- inverse(Tau[, ])
for(p in 1:nparasite){
  for(p.prime in 1:nparasite){
    rho[p,p.prime] <-
Sigma.Covar.raw[p,p.prime]/sqrt(Sigma.Covar.raw[p,p]*Sigma.Covar.raw[p.prime,p.prime])
  }
  sigma.sp[p] <- abs(xi.sp[p]) * sqrt(Sigma.Covar.raw[p,p])
  #scaling factor for scaled inverse Wishart
  xi.sp[p] ~ dunif(0,100)
}
#####
#Posterior predictive check
# Absolute residuals for logistic model
for (i in 1:nind){
  for (par in 1:nparasite){
    resid.infest_data[i, par] <- abs(InfestData[i, par] - p[i, par])
    Infest_sim[i, par] ~ dbern(p[i, par])
    resid.infest_sim[i, par] <- abs(Infest_sim[i, par] - p[i, par])
  }
}
fit_data <- sum(resid.infest_data[,])
fit_sim <- sum(resid.infest_sim[,])
test_fit <- step(fit_sim - fit_data)
}
"
, file=(model2bugs_malaria <- tempfile()))

```

```
#####
# RUN model via rjags
#####

param = c('b.island.star','b.habitat.star','b.hostsp.star','beta1','mu0','rho','test_fit')
na = 50000
nb = 250000
ni = 500000
nc = 2

rjags_malaria <- jags.model(model2bugs_malaria, data= data2bugs_malaria,
                           n.chains=nc , n.adapt = na)

update(rjags_malaria , nb)

out <- jags.samples(rjags_malaria, param, ni, thin=1000)

save(out, file="out-rjags_MalariaModel.RData")

#Gelman diagnostics for continuous estimates to check for mixture of chains
gelman.diag(out[["beta1"]])
gelman.diag(out[["mu0"]])
gelman.plot(out[["beta1"]])
gelman.plot(out[["mu0"]])

#Trace plots to individually check convergence for each parasite-covariate combination
plot(out$mu0[1, ,1], ylim = range(out$mu0), type='l')
lines(out$mu0[1, ,2], col =2)

plot(out$b.island.star[1,1, ,1], ylim = range(out$b.island.star), type='l')
lines(out$b.island.star[1,1, ,2], col =2)

###etc...for additional parasite/covariate combinations

##Check predictive fit ratio of 1's to 0's in the 1000 samples (500 per chain)
##Test fit of 1 indicates simulated residuals are greater than observed, 0 indicates the opposite
##A ratio of 0.4 to 0.6 indicates no discrepancy
sum(out$test_fit[, ,])/1000

#####
# Posterior processing
#####
#### Function to calculate posterior density credible intervals
hpd <- function(x, coverage)
{
  require(coda)
  x <- as.matrix(x)
  out <- matrix(NA, nrow=ncol(x), ncol=3)
  rownames(out) <- dimnames(x)[[2]]
  colnames(out) <- c("mode", "lower", "upper")
  f <- function(p) {
    if (p == density.range[2]) {
      set.coverage <- 0
    }
  }
  else {
    p.upper <- min(y.density$y[y.density$y > p])
  }
}
```

```

    p.lower <- max(y.density$y[y.density$y <= p])
    cov.upper <- sum(y.counts[y.density$y >= p.upper])/sum(y.counts)
    cov.lower <- sum(y.counts[y.density$y >= p.lower])/sum(y.counts)
    c <- (p.upper - p)/(p.upper - p.lower)
    set.coverage <- c * cov.upper + (1 - c) * cov.lower
  }
  return(set.coverage - coverage)
}
for (i in 1:ncol(x)) {
  y <- unclass(x[,i])
  y.density <- density(y, n=1024)
  m <- length(y.density$x)
  ## Find the mode
  out[i,1] <- y.density$x[which.max(y.density$y)]
  dx <- diff(range(y.density$x))/m
  breaks <- c(y.density$x[1] - dx/2, y.density$x + dx/2)
  y.counts <- hist(y, breaks=breaks, plot=FALSE)$counts
  density.range <- range(y.density$y)
  uniroot.out <- uniroot(f, density.range)
  ## Assuming that we have a single interval, find the limits
  out[i,2:3] <- range(y.density$x[y.density$y > uniroot.out$root])
  ## Check!
  if (sum(abs(diff(y.density$y > uniroot.out$root))) != 2) {
    warning("HPD set is not a closed interval for variable ",
            varnames(x)[i])
  }
}
return(out)
}

# Calculate posterior modes / 95% credible intervals for model parameters
##Change 'coverage' to 0.5 for 50% credible intervals if desired

##Calculate credible intervals for correlation matrix of parasite probabilities
Rho.95 <- array(NA, dim = c(nparasite, nparasite, 3))
for(i in 1:nparasite){
  for(j in 1:nparasite){
    Rho.95[i,j,] <- hpd(c(out$rho[i,j, , 1],
                        out$rho[i,j, , 2]), coverage = 0.95)  }
}

##Calculate credible intervals for intercept (mu0) and covariates
mu0.95 <- array(NA, dim = c(nparasite, 3))
for(i in 1:nparasite){
  mu0.95[i,] <- round(hpd(c(out$mu0[i, , 1],out$mu0[i, , 2]),
                        coverage = 0.95), 3)
}

##Inverse logit function translates mu0 estimates into prevalence estimates
inverse.logit<-function(x){
  exp(x)/(1+exp(x))
}

inverse.logit(mu0.95)

b.island.95 <- array(NA, dim = c(nparasite, nisland, 3))
for(i in 1:nparasite){

```

```

    for(s in 1:nisland){
      b.island.95[i,s,] <- round(hpd(c(out$b.island.star[i, s, , 1],
    out$b.island.star[i, s, , 2]), coverage = 0.95), 3)}
    }

##Exponentiate coefficients for covariates to translate estimates to odds ratios
exp(b.island.95)

###etc...for additional covariates

###Calculate proportion of variance explained for each covariate
## Combine chains for each covariate
library(abind)
Burn.island<-abind(out$b.island.star[, , 1],out$b.island.star[, , 2])
Burn.habitat<-abind(out$b.habitat.star[, , 1],out$b.habitat.star[, , 2])
Burn.hostsp<-abind(out$b.hostsp.star[, , 1],out$b.hostsp.star[, , 2])
Burn.propzos<-abind(out$beta1[, , 1],out$beta1[, , 2])
Burn.totzos<-abind(out$beta2[, , 1],out$beta2[, , 2])
Burn.mu0<-abind(out$mu0[, , 1],out$mu0[, , 2])

##Function to calculate squared values
make.power <- function(n) {
  pow <- function(x) { x^n}
  pow }
square <- make.power(2)

##Function to calculate proportion of environmental variance for each covariate
variance <- function(parasite, coverage)
{
  Variance <- array(NA, dim = c(4, 3))
  rownames(Variance)<-c('Island','Habitat','Host.species','RelAbundZos')
  island.var<-vector()
  habitat.var<-vector()
  hostsp.var<-vector()
  propzos.var<-vector()
  mu.var<-vector()
  Totalvar<-vector()
  FullVar<-matrix(NA,nrow=1000,ncol=4)
  colnames(FullVar)<-c('Island','Habitat','Host.species',
    'RelAbundZos')
  for(i in 1:1000){
    island.var[i] = square(sd(Burn.island[parasite,i]))
    habitat.var[i] = square(sd(Burn.habitat[parasite,i]))
    hostsp.var[i] = square(sd(Burn.hostsp[parasite,i]))
    propzos.var[i] = (Burn.propzos[parasite,i])*(Burn.propzos[parasite,i])
    Totalvar[i]=island.var[i]+propzos.var[i]+habitat.var[i]+hostsp.var[i]
    FullVar[i,1]=island.var[i]/Totalvar[i]
    FullVar[i,2]=habitat.var[i]/Totalvar[i]
    FullVar[i,3]=hostsp.var[i]/Totalvar[i]
    FullVar[i,4]=propzos.var[i]/Totalvar[i]
  }
  for(m in 1:4){
    Variance[m, ]=hpd(FullVar[, m], coverage=coverage)
  }
  return(Variance)
}

```



```
##Calculate proportions of environmental variance
h.zosvar.95<-variance(1, 0.95)

###etc... for additional parasites

#####
# Analyse variation in H/L ratios
#####

##Remove H/L ratio NAs
ZosDat2014<-ZosDat[ which(ZosDat$Data.source=="This study"),]
ZosDat2014<-ZosDat2014[!(is.na(ZosDat2014$H.L.Ratio)),]

##Logit transform H/L ratios, appropriate for skewed proportional data
HLratio.logit <- log((ZosDat2014$H.L.Ratio+0.00001)/ (1- (ZosDat2014$H.L.Ratio+0.00001)))

##Run linear regression using lmer
fullmod.logittrans <-lmer(HLratio.logit~ZosDat2014$Microfilaria+ZosDat2014$Haem*ZosDat2014$Plas+
                        ZosDat2014$Time+(1|ZosDat2014$Island)+(1|ZosDat2014$Species),
                        REML=F)

#Perform model comparisons, compute AIC scores
fullmod.logittrans.noMF <-lmer(HLratio.logit~ZosDat2014$Time+ZosDat2014$Haem*ZosDat2014$Plas+
                        (1|ZosDat2014$Island)+(1|ZosDat2014$Species),REML=F)
anova(fullmod.logittrans,fullmod.logittrans.noMF)

###etc... for additional models
```