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

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Summary

1. Experimental work increasingly suggests that non-random pathogen associations can affect the spread or severity of disease. Yet due to difficulties distinguishing and interpreting co-infections, evidence for the presence and directionality of pathogen co-occurrences in wildlife is rudimentary.

2. We provide empirical evidence for pathogen co-occurrences by analysing infection matrices for avian malaria (*Haemoproteus* and *Plasmodium* spp.) and parasitic filarial nematodes (microfilariae) in wild birds (New Caledonian *Zosterops* spp.).

3. Using visual and genus-specific molecular parasite screening, we identified high levels of co-infections that would have been missed using PCR alone. Avian malaria lineages were assigned to species level using morphological descriptions. We estimated parasite co-occurrence probabilities, while accounting for environmental predictors, in a hierarchical multivariate logistic regression.

4. Co-infections occurred in 36% of infected birds. We identified both positively and negatively correlated parasite co-occurrence probabilities when accounting for host, habitat and island effects. Two of three pairwise avian malaria co-occurrences were strongly negative, despite each malaria parasite occurring across all islands and habitats. Birds with microfilariae had elevated heterophil to lymphocyte ratios and were all co-infected with avian malaria, consistent with evidence that host immune modulation by parasitic nematodes facilitates malaria co-infections. Importantly, co-occurrence patterns with microfilariae varied in direction among avian malaria species; two malaria parasites correlated positively but a third correlated negatively with microfilariae.

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

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

Introduction

How pathogens are distributed and how changing environments cause disease spillover across species or geographic barriers are key questions in ecology (Wood *et al.* 2012; Hoberg & Brooks 2015; Plowright *et al.* 2015; Wells *et al.* 2015). While the environment undoubtedly influences pathogen infections (Budria & Candolin 2013; Sehgal 2015), hosts often carry multiple pathogens whose interactions can alter infection dynamics (Cattadori, Boag & Hudson 2008; Johnson & Hoverman 2012). Infection with one pathogen can increase a host's susceptibility to other pathogens or to harmful disease (Bordes & Morand 2011). For example, chickens infected with *Staphylococcus aureus* bacteria develop more severe disease when

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inoculated with influenza than those without co-occurring bacteria (Kishida *et al.* 2004). Pathogen interactions might also be antagonistic. In leaf-cutting ants, competition between fungal pathogen strains leads to decreased overall pathogen transmission (Hughes *et al.* 2004). Yet while interactions such as competition and facilitation form the foundations of ecology (Dayton 1971), detecting wildlife pathogen associations is challenging due to (i) difficulties distinguishing co-infections (Valkiūnas *et al.* 2006; Tompkins *et al.* 2011) and (ii) a lack of statistical approaches to disentangle environmental predictors (Muturi *et al.* 2008; Fenton *et al.* 2014). Hierarchical multivariate approaches overcome this hurdle by assessing both environmental influences and interspecific co-occurrences in joint distribution models (Ovaskainen, Hottola & Siitonen 2010; Kissling *et al.* 2012). We use one such tool, multivariate logistic regression, to describe the presence and directionality of blood parasite co-occurrences in wild birds.

Haematozoan blood parasites, including haemosporidians (*Plasmodium* and *Haemoproteu*s spp.; collectively referred to here as 'malaria' parasites to avoid confusing 'haemosporidian' and 'haematozoan') and microfilaria (blood stages of filarial nematodes), are vector-transmitted parasites that often exist in co-infection (Bush 2001; Atkinson, Thomas & Hunter 2008; Astudillo *et al.* 2013; Clark, Clegg & Lima 2014). Because both parasites are important disease agents, understanding factors that drive their transmission and occurrence is vital to unravel their impacts on hosts (Muturi *et al.* 2008; Griffiths *et al.* 2015). Haematozoans are strongly driven by environmental factors, such as temperature and habitat, that can limit parasite development or vector distributions (Rogers *et al.* 2002; Santiago-Alarcon, Palinauskas & Schaefer 2012; Freed & Cann 2013; Sehgal 2015). However, haematozoan infections may also be influenced by biotic parasite interactions (Su *et al.* 2005; Telfer *et al.* 2010). Experimental work in mammals shows that parasitic nematodes can modulate immune responses of hosts by depressing antigen-recognizing lymphocytes while increasing neutrophils, potentially increasing concomitant malaria transmission (Nacher *et al.* 2001; Graham *et al.* 2005; Su *et al.* 2005; Muturi *et al.* 2008). Competition between malaria strains can also occur and is likely to influence within-host progression (Bell *et al.* 2006). Yet despite increasing evidence for parasite associations in model mammalian hosts (Telfer *et al.* 2010; Fenton *et al.* 2014), evidence from non-model hosts is primarily experimental and remains limited by a paucity of co-infection data (Jackson *et al.* 2006; Knowles 2011; Tompkins *et al.* 2011).

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

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

Materials and methods

FIELD SAMPLING AND LABORATORY METHODS

New Caledonia is a subtropical Pacific archipelago consisting of four main islands (Fig. 1a). The archipelago supports four *Zosterops* spp., including the regionally widespread *Zosterops lateralis*, the New Caledonian endemic *Zosterops xanthochrous*, and two single-island endemics, *Zosterops minutus* and *Zosterops inornatus* (both of which only occur on the island of Lifou; Dutson 2012). All four species are omnivorous passerines that occur in mixed-species flocks. We captured *Zosterops* spp. with mist nets on the four main islands from January to March 2014. Sites were chosen to represent the three primary forested habitats in New Caledonia, namely dry lowland forest (Grand Terre, Ouvéa), lowland rain forest (Ouvéa, Lifou and Maré) and montane rain forest (Grand Terre; see Fig. S1, Supporting Information for site map). Blood samples were collected from each bird ($n = 275$). Blood smears were also taken for 245 birds.

Avian malaria PCR screening and sequencing followed Clark *et al.* (2015), with the following variations. Sequences suggested amplification bias towards *Plasmodium* spp. when co-occurring with *Haemoproteus* spp., with clean *Plasmodium* sequences (i.e. absence of double peaks) retrieved from 16 confirmed *Plasmodium*/*Haemoproteus* co-infections (see below for smear screening). Eight known co-infections produced *Haemoproteus* sequences, while a further six produced double peaks (re-sequencing of all six producing clean *Plasmodium* sequences). *Haemoproteus* lineages were therefore characterized using genus-specific primers designed from sequences recovered in Australasian hosts (Clark & Clegg 2015; Clark, Clegg & Klaassen 2016). These primers successfully amplified *Haemoproteus* DNA from all visually observed *Plasmodium*/*Haemoproteus* co-infections. A Bayesian 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 782 bp of the parasite large subunit rDNA. GenBank accessions for parasite lineages are KX604232 – KX604237. Malaria lineages are also deposited in the MalAvi data base (Bensch,

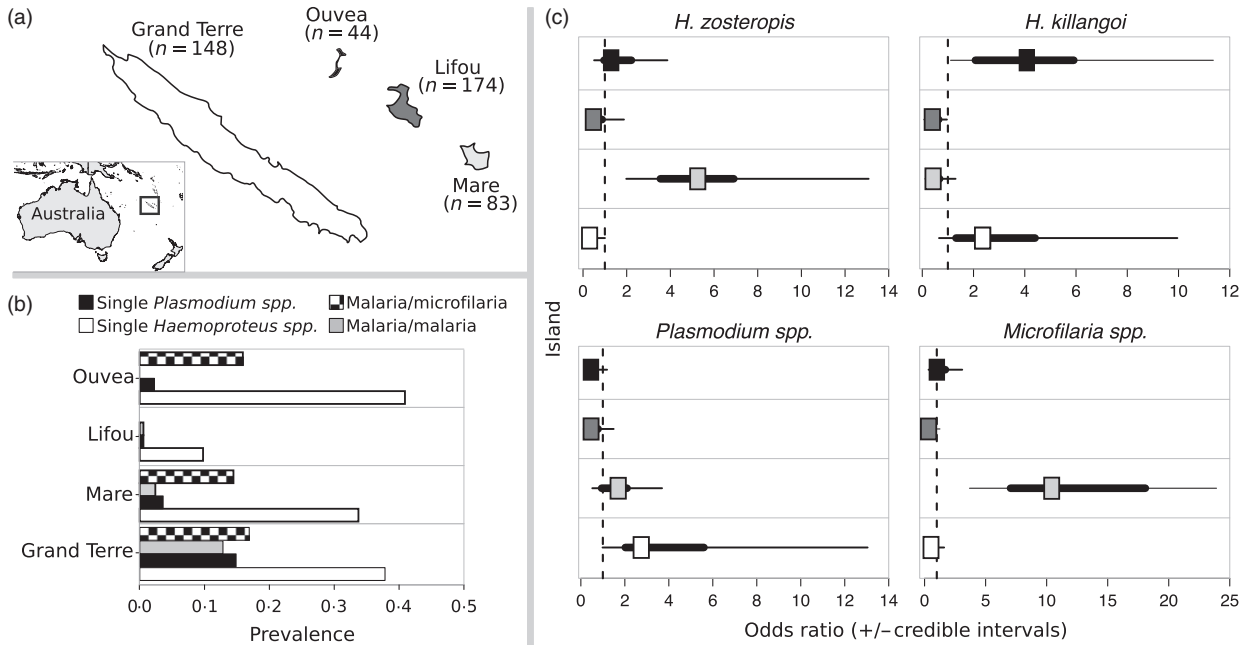

Fig. 1. (a) *Zosterops* spp. sample sizes (*n*) on New Caledonian islands. (b) Observations of haematozoan parasite infections and co-infections. Note that only 275 samples were screened for microfilaria. (c) Estimated odds ratios of infection probability across islands. Presented are posterior modes, 50% highest posterior density credible intervals (CI) (thick lines) and 95% highest posterior density CI (thin lines). Colours of symbols correspond to colours of islands in (a).

Hellgren & Pérez-Tris 2009). PCR protocols, phylogenetic methods and the malaria consensus phylogeny (Fig. S2) are available from the Dryad Digital Repository (doi: 10.5061/dryad.pp6k4).

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 characterize 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 1000× to identify malaria parasites and to calculate H/L ratios by categorizing 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 data set 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 (*Haemoproteus zosteropis*, *Haemoproteus killangoi*, *Plasmodium* spp. and microfilaria; see supporting information for descriptions and molecular barcoding

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

	Grand Terre	Maré	Ouvéa	Lifou
(*a*) *Zosterops* host species				
Zosterops lateralis	10 *(26)*	5 *(20)*	44 *(0)*	27 *(20)*
Zosterops xanthochrous	69 (*43*)	38 (*20*)	Absent	Absent
Zosterops minutus	Absent	Absent	Absent	72 *(25)*
Zosterops inornatus	Absent	Absent	Absent	10 *(20)*
(*b*) Haematozoan parasites				
Haemoproteus zosteropis	60	36	14	9
Haemoproteus killangoi	28	5	11	8
Plasmodium spp.	76	7	3	2
Microfilaria	25	12	7	0

(c) Observed co-infections	*Plasmodium* spp.	Microfilaria	*H. killangoi*
H. zosteropis	28	33	2
H. killangoi	2	1	–

of *H. zosteropis* and *H. killangoi*) across 17 sites [46 birds in montane rain forest (Grand Terre), 111 in open lowland forest (Grand Terre and Ouvéa) and 292 in lowland rain forest (Maré, Lifou and Ouvéa); Figs 1a and 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 were 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 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. zosteropis* and *H. killangoi* to asexually develop. *Zosterops* spp. sample sizes ranged from three to 105 and proportional abundance ranged from 19·4% to 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:

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

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

$$\begin{aligned}\text{logit}(\Psi(p,i)) = {} & \beta_0^P + \beta_{\text{HostSp}}^P(i) + \beta_{\text{Island}}^P(s) + \beta_{\text{Forest}}^P(s) \\ & + \gamma_{\text{A}}^P A_{\text{zost.scale}}(s) + E(p,i) \qquad \text{eqn 2}\end{aligned}$$

Here, β_0^P is the parasite-specific intercept, while coefficients β_{HostSp}^P, β_{Island}^P and β_{Forest}^P estimate variation in infection probability due to host species, island and forest type, respectively (categorical variables; β-values estimated for each level). Superscript '^{P}' is used as coefficients were estimated independently for each parasite species. Coefficient γ_{A}^P estimates the effect of *Zosterops* proportional abundance A_{zost}, estimated as proportion of *Zosterops* individuals from all captured birds at each site. To account for unequal sampling across sites, we modelled A_{zost} as a binomial function of total mist net captures (all species; N_{total}) and *Zosterops* spp. total abundance N_{zost}:

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

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

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:

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

Here, Ω 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 β^P coefficients in eqn. 2. The two parameters of the inverse Wishart are degrees of freedom d.f. and a positive-definite scale matrix of dimension $p \times p$ (p = total number of parasite species). We set d.f. = $p + 1$ 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 $A_{\text{zost}}(s)$, we used a *Beta*(*2,2*) distribution truncated between 0·05 and 0·9 (based on observed range limits for $A_{\text{zost}}(s)$). For categorical covariates (β_{HostSp}^P, β_{Island}^P and β_{Island}^P), we used redundancy coefficients to improve convergence and scale estimates (Gelman & Hill 2007). For example, coefficient $\beta_{\text{HostSp}}^{P\,*}$ was calculated for parasite species p in host species h as:

$$\beta_{\text{HostSp}}^{P\,*}(h) = \beta_{\text{HostSp}}^P(h) - \text{mean}(\beta_{\text{HostSp}}^P)$$

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 data set, 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. zosteropis* 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 credible intervals (CI). We used odds ratios (OR) to compare strength of change in infection probabilities for levels of categorical covariates. We considered CI that did not overlap with zero or with those from other covariates as 'significant'.

ANALYSIS OF HOST HETEROPHIL TO LYMPHOCYTE RATIOS

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

Data were analysed in R version 3.2.1 (R Core Team 2008). Data and R code used to perform analyses are presented in supporting information and are available from the Dryad Digital Repository: (doi: 10.5061/dryad.pp6k4).

Results

ENVIRONMENTAL INFLUENCES ON PARASITE INFECTION PROBABILITIES

In total, 228 of 449 *Zosterops* individuals were infected with haematozoans, including 191 *Haemoproteus*, 88 *Plasmodium* and 41 microfilaria infections (Table 1b; Fig. 1a, b). Nine avian malaria lineages were morphologically identified to species level for the first time, including three lineages of *H. killangoi* and four of *H. zosteropis* (Figs S2–S4). Each of the four focal parasites occurred on all islands, with the exception of microfilariae (absent from Lifou; Table 1b; Fig. 1b). The multivariate logistic regression obtained good fit (Bayesian $P = 0{\cdot}56$). Estimated prevalence across all individuals (β_0) was highest for *H. zosteropis* (CI: 14–45%), followed by microfilaria (5–22%), *Plasmodium* spp. (4–18%) and *H. killangoi* (2–11%).

'Forest type' explained 15–63% of environmental variation in occurrence probability for microfilaria, 3–65% for *Plasmodium* spp. and 1–28% for *H. zosteropis*, with each parasite less likely to occur in montane rain forest than the two lowland forest categories (OR: 0·02–0·27 for microfilaria, 0·05–0·65 for *Plasmodium* spp. and 0·04–0·75 for *H. zosteropis*). Infection patterns differed across lowland forest categories, with *H. zosteropis* and microfilaria more likely to occur in lowland rain forest (OR: 2·1–13·8 and 2·1–12·8, respectively) and *Plasmodium* spp. infections more likely in open lowland forest (OR: 2·1–14·5).

'Island' explained 7–53% of environmental variance in occurrence probability for microfilaria, 2–28% for *H. zosteropis* and 1–68% for *H. killangoi*. Both *H. zosteropis* and microfilaria were more likely on Maré than remaining islands (OR: 3·7–37·3 and 1·9–13·1, respectively; Fig. 1c). Infections with *H. killangoi* were more likely on Ouvéa (OR: 1·1–12·1; Fig. 1c). In addition to island and habitat effects, *H. zosteropis* occurrence was negatively influenced by *Zosterops* spp. '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 CIs 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. zosteropis*/*Plasmodium*/Microfilaria triple infections and one *H. killangoi*/*H. zosteropis*/*Plasmodium* triple infection. After accounting for environmental covariates, estimated covariances revealed 'significantly' correlated infection probabilities for all parasite pairs apart from *H. zosteropis*/*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. zosteropis*, but negatively with *H. killangoi* (Fig. 2). In fact, thirty-three of 44 observed microfilaria infections co-occurred with *H. zosteropis*, 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 (ΔAIC without microfilaria: +11·17; Fig. 3). This elevation was driven by increased heterophils (mean with microfilaria: $12{\cdot}73 \pm 2{\cdot}21$; without: $5{\cdot}03 \pm 0{\cdot}45$) and decreased lymphocytes (mean with microfilaria: $74{\cdot}93 \pm 2{\cdot}28$; without: $82{\cdot}68 \pm 0{\cdot}85$). Neither *Haemoproteus* nor *Plasmodium* spp. influenced H/L ratios, either as separate variables or combined (ΔAIC without *Haemoproteus*: −2·91; without *Plasmodium*: −2·82; without 'malaria': −1·11; Fig. 3).

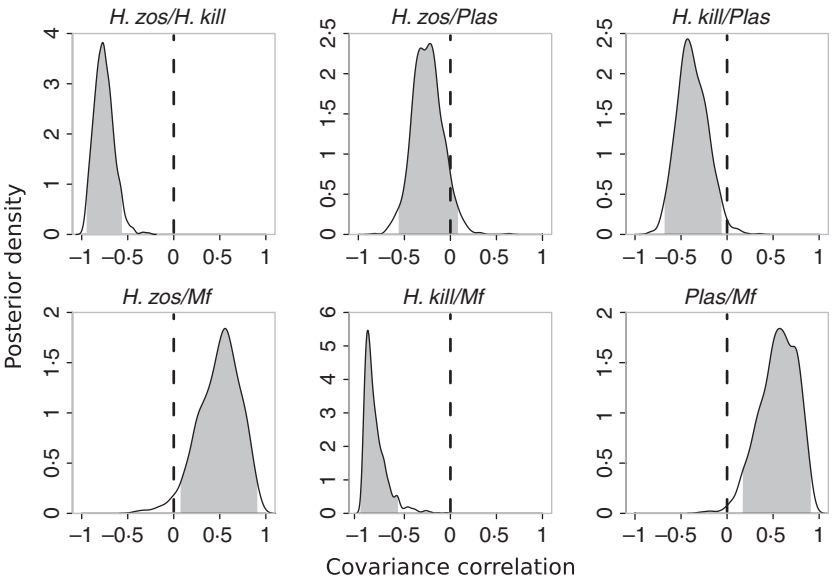

Fig. 2. Haematozoan parasite pairwise correlations of infection probabilities. Correlations were estimated from a parasite variance–covariance matrix after accounting for environmental covariates in a multivariate logistic regression. Shading indicates 95% highest posterior density credible intervals. *Plas.*, *Plasmodium* spp.; Mf., microfilaria.

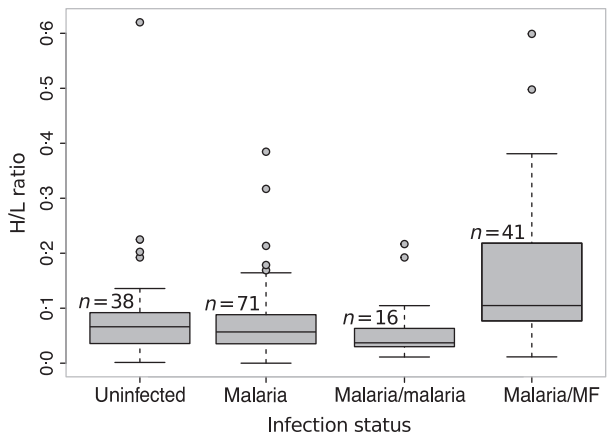

Fig. 3. Heterophil to lymphocyte ratios for *Zosterops* spp. across parasite infection classes. Also presented are total sample sizes (*n*) for each infection. MF, microfilaria.

Discussion

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

CORRELATED INFECTION PROBABILITIES: EVIDENCE OF PARASITE COMPETITION AND FACILITATION?

We identified negative parasite co-occurrence probabilities between *H. zosteropis*/*H. killangoi* and between *H. killangoi* /*Plasmodium* spp., supporting our prediction that interspecific malaria infections would be negatively correlated. Only two co-infections were observed for each of the above parasite pairs, despite each parasite occurring on all islands and habitats. Considering that *H. zosteropis* and *H. killangoi* are avian host specialists that appear restricted to Zosteropidae (Valkiūnas 2005; Clark & Clegg 2015), our results may be evidence of interspecific competition. We also found a striking difference in likelihoods of microfilaria co-infection for the two *Haemoproteus* species. We predicted malaria infections would positively correlate with microfilaria; yet, while no filarial parasites occurred in birds free from avian malaria, birds carrying *H. killangoi* rarely carried microfilaria. In comparison, birds carrying *H. zosteropis* had increased likelihood of carrying microfilaria when accounting for their similar environmental affiliations. Contrasting patterns for host-specialist *Haemoproteus* parasites suggest associations with immune-modulating nematodes are uneven between rival malaria species, a fascinating finding that deserves further attention in field and laboratory studies.

Explaining patterns of co-occurrence for vector-borne parasites requires careful consideration of the role of vectors. Similarly to previous studies, we found important environmental influences on blood parasite distributions (Lachish *et al.* 2011; Oakgrove *et al.* 2014; Sehgal 2015). Despite wide CIs owing to uncertainty, we identified habitat and island infection patterns that likely reflect distributions of arthropod vectors (Rogers *et al.* 2002; Santiago-Alarcon, Palinauskas & Schaefer 2012). Both *Haemoproteus* and microfilaria are known to use Ceratopogonid

midges as vectors, and evidence suggests that different *Haemoproteus* parasites can use different Ceratopogonid species (Santiago-Alarcon, Palinauskas & Schaefer 2012). Associations between *H. zosteropis* and microfilaria could be evidence of a shared vector, while a different vector may transmit *H. killangoi*, perhaps reducing co-infections. This hypothesis adds to the growing need for future studies of haematozoan vectors (Clark, Clegg & Lima 2014; Bobeva *et al*. 2015; Žiegytė & Valkiūnas 2015; Bernotienė & Valkiūnas 2016). In addition to environmental effects, a surprising finding was the negative influence of *Zosterops* spp. proportional abundance on *H. zosteropis* occurrence. The idea that hosts reach higher abundance where infections are lower touches on exciting evolutionary questions, such as host–parasite interactions driving taxon cycles (Ricklefs *et al*. 2016) or shaping host dispersal patterns (Poulin *et al*. 2012; Aharon-Rotman *et al*. 2016).

Our data were not complete, as only samples from 2014 were subject to smear and genus-specific PCR screening, adding to uncertainty in our estimates and emphasizing the need for greater scrutiny of co-occurring wildlife pathogens (Petney & Andrews 1998; Knowles 2011; Meixell *et al*. 2016). In addition to incomplete data, some parasite associations seen here could have been inflated by missing covariates (Pollock *et al*. 2014), as we lacked microhabitat data such as temperature and moisture that can influence local transmission (Zamora-Vilchis, Williams & Johnson 2012; Cornuault *et al*. 2013; Sehgal 2015; Wilkinson *et al*. 2016). Due to complex environmental influences and the inherent uncertainty in pathogen observations, we propose that multivariate logistic regression combined with appropriate covariate data provides a useful platform to detect wildlife pathogen associations.

ALTERED HETEROPHIL TO LYMPHOCYTE RATIOS IN MALARIA/MICROFILARIA COINFECTIONS

Though often overlooked, haematozoan co-infections are important, as they may compound effects on host condition and survival (Valkiūnas *et al*. 2006; Palinauskas *et al*. 2011; Oakgrove *et al*. 2014; Dimitrov *et al*. 2015). Yet identifying mechanisms that drive wildlife parasite associations is challenging (Cattadori, Boag & Hudson 2008; Tompkins *et al*. 2011). Our finding of altered H/L ratios during microfilaria infection identifies immune modulation as a possible mechanism by which parasitic nematodes may facilitate co-occurring malaria. Microfilariae led to decreased lymphocytes and increased heterophils, changes that could decrease a host's ability to regulate pathogens through antigen recognition (Pedersen & Fenton 2007; Bordes & Morand 2011). We did not observe changes in H/L ratios in birds carrying malaria but not microfilaria, consistent with prior studies (Ricklefs & Sheldon 2007) and suggesting the presence of parasitic nematodes drove these changes. This pattern supports laboratory evidence that microfilariae depress adaptive immune pathways responsible for identifying infections while increasing neutrophil-associated inflammation (Druilhe, Tall & Sokhna 2005).

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

CONCLUSIONS

We present evidence that biotic associations play important roles in the occurrences and infection likelihoods of haematozoan parasites. Our description of parasite co-occurrence patterns provides critical new insights into disease ecology, as parasite associations are expected across many host systems (Bell *et al*. 2006; Pérez-Tris *et al*. 2007; Johnson & Buller 2011; Vaumourin *et al*. 2015), yet evidence from wildlife is biased towards mammalian hosts (Lello *et al*. 2004; Tompkins *et al*. 2011; Hellard *et al*. 2015). Additionally, we show that co-infections are difficult to identify using PCR alone, a finding demonstrated for many host–pathogen systems (Valkiūnas *et al*. 2006; Dyachenko *et al*. 2010; Grybchuk-Ieremenko *et al*. 2014; Moustafa *et al*. 2016). We overcame this hurdle by combining traditional and molecular parasitology methods, a multidisciplinary approach that we recommend for future work on wildlife co-infections.

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Data accessibility

Malaria lineages are deposited in GenBank (accession numbers: KX604232 – KX604237) and the MalAvi data base. Microfilaria LSU

lineages are deposited in GenBank (accession numbers: KX604238 – KX604240). Data and R code used for analyses are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.pp6k4 (Clark *et al*. 2016).

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Supporting Information

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

Appendix S1. Sample sites, PCR and phylogenetic methods, and parasite descriptions.

Appendix S2. Sample R Code used for statistical analyses.

Appendix S3. Raw data used for statistical analyses.