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

Nicholas J. Clark¹,²*, Konstans Wells¹, Dimitar Dimitrov³ and Sonya M. Clegg¹,⁴

¹Environmental Futures Research Institute, School of Environment, Griffith University, Gold Coast, Qld 4111, Australia; ²Natural Environments Program, Queensland Museum, Institute of Biodiversity and Ecosystem Research, P.O. Box 3300, South Brisbane, Qld 4101, Australia; ³Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences, 2 Gagarin Street, Sofia 1113, Bulgaria; and ⁴Department of Zoology, Edward Grey Institute of Field Ornithology, University of Oxford, Oxford OX1 3PS, UK

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). 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...
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 (Valkiunas 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, Sotola & 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 Haemoproteus 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 parasite 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 accesses for parasite lineages are KX604232 – KX604237. Malaria lineages are also deposited in the MalAvi data base (Bensch, © 2016 The Authors. Journal of Animal Ecology © 2016 British Ecological Society, Journal of Animal Ecology
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 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. zosterops 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; rather, they represent the tendency of parasites to co-occur more or less than expected by chance (based on residual correlations), after accounting for environmental $\beta^2$ coefficients in eqn 2. The two parameters of the inverse Wishart are degrees of freedom $\nu$ and a positive-definite scale matrix of dimension $p \times p$ ($p = \text{total number of parasite species}$). We set $\nu = p + 1$ 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 $A_{\text{host}}(s)$, we used a Beta(2,2) distribution truncated between 0.05 and 0.9 (based on observed range limits for $A_{\text{host}}(s)$). For categorical covariates ($\beta^2_{\text{hostSp}}, \beta^2_{\text{island}}$ and $\beta^2_{\text{forest}}$), we used redundancy coefficients to improve convergence and scale estimates (Gelman & Hill 2007). For example, coefficient $\beta^2_{\text{hostSp}}$ was calculated for parasite species $p$ in host species $h$ as:

$$\beta^2_{\text{hostSp}}(p, h) = \beta^2_{\text{hostSp}}(h) - \text{mean}(\beta^2_{\text{hostSp}})$$

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 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. zosterops 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 lineage 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 $P = 0.56$). Estimated prevalence across all individuals ($\beta_0$) was highest for *H. zosteropis* (CI: 14–45%), followed by microfilarial (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-OCURRENCE 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 pair wise 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 ($\Delta$AIC without microfilaria: +11.17; Fig. 3). This elevation was driven by increased heterophils (mean with microfilaria: 12.73 ± 2.21; without: 5.03 ± 0.45) and decreased lymphocytes (mean with microfilaria: 74.93 ± 2.28; without: 82.68 ± 0.85). Neither *Haemoproteus* nor *Plasmodium* spp. influenced H/L ratios, either as separate variables or combined ($\Delta$AIC without *Haemoproteus*: −2.91; without *Plasmodium*: −2.82; without ‘malaria’: −1.12; Fig. 3).

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.

**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 (Valkiunas 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. kil languis*, 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; Ziegert & Valkiūnas 2015; Bernotienė & Valkiūnas 2016). In addition to environmental effects, a surprising finding was the negative influence of *Zostera* 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 (Drulhe, Tall & Sokhma 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 (Metcalfe 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
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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.