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Prevalence and diversity patterns of avian blood parasites in degraded African rainforest habitats

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Abstract

Land use changes including deforestation, road construction and agricultural encroachments have been linked to the increased prevalence of several infectious diseases. In order to better understand how deforestation affects the prevalence of vector-borne infectious diseases in wildlife, nine paired sites were sampled (disturbed vs. undisturbed habitats) in Southern Cameroon. We studied the diversity, prevalence and distribution of avian malaria parasites (*Plasmodium* spp.) and other related haemosporidians (species of *Haemoproteus* and *Leucocytozoon*) from these sites in two widespread species of African rainforest birds, the yellow-whiskered greenbul (*Andropadus latirostris*, Pycnonotidae) and the olive sunbird (*Cyanomitra olivacea*, Nectariniidae). Twenty-six mitochondrial cytochrome *b* lineages were identified: 20 *Plasmodium* lineages and 6 *Haemoproteus* lineages. These lineages showed no geographic specificity, nor significant differences in lineage diversity between habitat types. However, we found that the prevalence of *Leucocytozoon* and *Haemoproteus* infections were significantly higher in undisturbed than in deforested habitats (*Leucocytozoon* spp. 50.3% vs. 35.8%, *Haemoproteus* spp. 16.3% vs. 10.8%). We also found higher prevalence for all haemosporidian parasites in *C. olivacea* than in *A. latirostris* species (70.2% vs. 58.2%). Interestingly, we found one morphospecies of *Plasmodium* in *C. olivacea*, as represented by a clade of related lineages, showed increased prevalence at disturbed sites, while another showed a decrease, testifying to different patterns of transmission, even among closely related lineages of avian malaria, in relation to deforestation. Our work demonstrates that anthropogenic habitat change can affect host–parasite systems and result in opposing trends in prevalence of haemosporidian parasites in wild bird populations.

Keywords: *Andropadus latirostris*, Cameroon, *Cyanomitra olivacea*, deforestation, *Haemoproteus*, *Leucocytozoon*, malaria, *Plasmodium*

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Introduction

As human populations increase worldwide, landscapes are being transformed at unprecedented rates. These land use changes include deforestation, road construction, agricultural encroachment and the expansion of urban environments. Such modifications lead not only to forest fragmentation and loss of biodiversity (Fahrig 2003; Foley *et al.* 2005; Tischendorf *et al.* 2005; Devictor *et al.* 2008) but can also alter ecological and evolutionary processes (Ryall & Fahrig 2006; Malanson *et al.* 2007; Smith *et al.* 2008), including those between hosts and parasites (Walsh *et al.* 1993). These habitat changes can potentially favour or eliminate parasites depending on the availability of their host, and in some cases introduce novel pathogens to these changing environments. Land use changes can thus upset natural ecological dynamics, resulting in an increase in disease outbreaks, emergence events, and modifications of the transmission of endemic pathogens (Patz *et al.* 2000). Current research has linked forest fragmentation, urban sprawl

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and agricultural expansion to several infectious diseases including Lyme disease in the Northeast United States (LoGiudice *et al.* 2003), Nipah virus in Malaysia (Chua *et al.* 1999) and human malaria (Tadei *et al.* 1998; Vittor *et al.* 2006). Furthermore, it is estimated that 75% of human diseases have their origin as zoonotic pathogens and can be linked to either wildlife or domestic animals (Taylor *et al.* 2001; Jones *et al.* 2008). As such, it has become essential to discern how land-cover changes affect human and animal epidemiology in order to better contain and prevent the spread of infections.

In addition to land cover changes, many diseases and pathogens are known to be sensitive to climatic conditions. For example, meteorological factors have been found to be important drivers of vector-borne disease transmission (Harvell *et al.* 2002). Factors such as temperature, rainfall and humidity have been associated with the dynamics of malaria vector populations and therefore with the spread of the disease (Hay & Lennon 1999; Ye *et al.* 2007). For example, recent work in Kenya (Afrane *et al.* 2008) linked microclimatic changes caused by deforestation to the increased survival and fecundity of the mosquito *Anopheles gambiae.* Thus infectious disease prevalence may differ with habitat change and associated altered microclimates.

Here we focus on the blood parasites in two African bird species the yellow-whiskered greenbul (*Andropadus latirostris,* Pycnonotidae) and the olive sunbird (*Cyanomitra olivacea,* Nectariniidae), both of which occupy distinct ecological niches yet share the same habitat. These bird species are common throughout Western Africa, and are found in both disturbed and undisturbed habitats (Borrow & Demey 2005). Haemosporidian parasites (Haemosporida) of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are transmitted by diverse and widely distributed blood-sucking dipteran insects (Valkiūnas 2005). The primary vectors of avian malaria parasites (*Plasmodium* spp.) are Culicidae mosquitoes belonging to eight genera, but particularly species of *Culex* and *Culiseta* (Valkiūnas 2005). *Haemoproteus* spp. of songbirds are transmitted by blood-sucking biting midges of the *Culicoides* (Valkiūnas & Iezhova 2004) and *Leucocytozoon* spp. are transmitted by black flies belonging to the Simuliidae (Forrester & Greiner 2009).

Several studies have begun to address the relationship between avian haemosporidian parasites and various ecological determinants (Valkiūnas 2005). For example, Wood *et al.* (2007) have shown marked and complex associations between avian malaria infections and landscape on a local scale, within a single population of birds. In Africa, much less is known about the ecology of avian haemosporidians. There has been extensive work conducted to determine the prevalence and diversity of these parasites in rainforest birds in West-Central Africa (Waldenström *et al.* 2002; Sehgal *et al.* 2005; Valkiūnas *et al.* 2005; Beadell *et al.* 2009). However, few studies have focused on the interplay between the distribution of haemosporidian infections and deforestation. A recent study examining prevalence of infection in both pristine and disturbed forests from a limited sampling over a broad geographic range in Cameroon found a higher prevalence of *Plasmodium* lineages in pristine as compared to disturbed sites (Bonneaud *et al.* 2009). Other research examining prevalences in this region of Africa did not show marked differences in prevalence of haemosporidians between seasons or years (Sehgal *et al.* 2005). This region of Africa is ideal to investigate the effects of habitat modification due to continued high rates of deforestation and forest fragmentation activities (FAO 2007).

We designed our study by selecting numerous paired forested sites that were close enough to each other to share similar environmental metrics, but differ in the degree of habitat modification. Here, we examined how diversity and prevalence of haemosporidian parasites differ between disturbed and undisturbed habitat types and between two bird species. Different bird species have been found to possess varying assemblages of parasite communities (Ricklefs *et al.* 2005; Arriero & Moller 2008), but there is little empirical data on blood parasite diversity for individual bird species over a large scale in Africa, particularly in relation to human-induced habitat change. Results revealed significant alterations in patterns of hemosporidian parasite prevalence and species composition associated with deforestation.

Methods

Site selection and characterization

Fieldwork took place between June and December in both 2005 and 2007 at 19 sites across the southern region of Cameroon (Fig. 1). Site selection involved the delineation of nine block areas, with two, or in one case three, sites chosen from each block. From each block, one site was selected for being 'disturbed', defined as having anthropogenic disturbances or activities (agriculture preceded by logging), and the other site was 'undisturbed', defined as primary forested areas exhibiting very little or no human activity. These sites were specifically selected so that side-by-side comparisons could be conducted to examine the prevalence of malaria and other haemosporidian parasites in two distinct habitat types, while remaining within the same ecocline. The maximum distance between each site of a pair was 18 km, with an average distance of 7.1 km (Supporting information, Table S1). The three

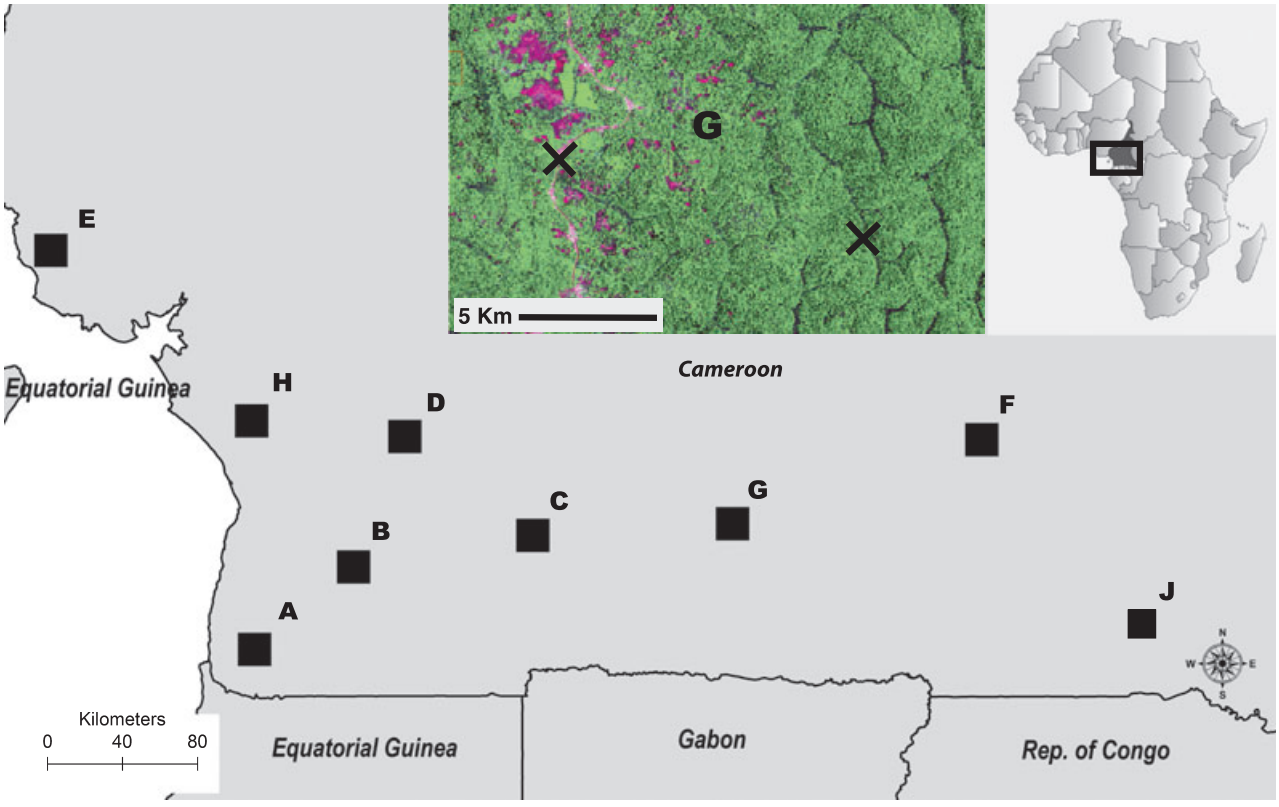

Fig. 1 Map showing the geographic distribution of the study sites (A–J), each letter corresponding to a paired site. Black squares designate the paired site locations. Inset shows an ASTER (Advanced Spaceborne Thermal Emission and Reflection Radiometer) image, with visible and near-infrared (VNIR) bands at 15 m resolution, year 1999. Black crosses represent the two collection sites at paired site G (Bitye and Mvono). In this pseudo-natural image, green shows vegetation and purple represents disturbances.

sites of J include one undisturbed and two disturbed sites. For purpose of analysis we pooled the two disturbed sites (63 km apart) for comparison with the single undisturbed site located 150 km away (these three sites are located in a remote area of southeast Cameroon and are situated within a contiguous rainforest tract with similar rainfall patterns). To aid in site selection while in the field, we used NASA's multispectral mosaic of orthorectified Landsat7 ETM+ images from the year 2000 at 90 m resolution. ASTER (Advanced Spaceborne Thermal Emission and Reflection Radiometer) images were obtained from the U.S. Geological Survey.

Field methods

Bird sampling targeted two species, the yellow-whiskered greenbul and the olive sunbird, which were found at all 19 locations. These two species are nonmigratory; the olive sunbird is known to be mainly sedentary and prefer to forage in the lower strata (0–6 m) (Cheke *et al.* 2001) however little is known about the movement patterns of the yellow-whiskered greenbul in this part of Africa. Both of these species can be commonly found in secondary forest and agroforestry systems (Bobo 2007). We captured and investigated 851 birds (Table 1), which were identified according to Borrow & Demey (2005). To

Table 1 Sample numbers for each species, and numbers of infected samples by habitat type for *Haemoproteus*, *Plasmodium* and *Leucocytozoon* spp. as assessed by PCR

		Habitat (*n*)		*Haemoproteus* spp.		*Plasmodium* spp.		*Leucocytozoon* spp.	
Species	*N*	Undisturbed	Disturbed	Undisturbed	Disturbed	Undisturbed	Disturbed	Undisturbed	Disturbed
Cyanomitra olivacea	440	166	274	38	40	55	92	98	111
Andropadus latirostris	411	202	209	22	12	49	71	87	61
Total	851	368	483	60	52	104	163	185	172

capture birds at each site mist nets were erected; 12 to 20 mist nets (12 m long and 2.6 m high with 32 × 32 mm mesh). Once captured, all birds were measured, weighed and banded using methods described in Smith *et al.* (1997). Recaptured birds were not used in this study. We obtained 5–20 μL of blood from each bird by venepuncture from the ulnar (brachial) vein. The blood was immediately stored in lysis buffer (10 mM Tris-HCL pH 8.0, 100 mM EDTA, 2% SDS).

Parasite screening using microscopy

From each bird, two or three blood films were prepared using freshly drawn blood. Blood films were air-dried within 5–15 s after their preparation. Because of the humid environment, we used a battery-operated fan to aid in the drying of the blood films. Smears were fixed in absolute methanol for 1 min on the day of their preparation; they were air-dried and packed into paper bands to avoid potential contamination. The blocks of slides were then wrapped in paper and kept in sealed plastic packs. In the laboratory, the blood films were stained in a 10% working stock solution of Giemsa stain, pH 7.0–7.2, at 18–20 °C for 1 h. All blood films were stained between 5 and 30 days after their fixation. One blood film from each individual was examined using an Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE. Approximately 100 fields at both low (×400) and high magnification (×1000) were viewed. In total, the approximate number of screened red blood cells was 5×10^5 in each blood film. Only good quality slides, i.e., without any features of lysis of cells and well-stained blood cells and parasites, were used for microscopic examination. Details of preparation, staining and microscopic examination of blood films were described by Valkiūnas *et al.* (2008a).

Parasite screening using PCR

Parasite DNA was extracted from whole blood using the DNeasy kit (Qiagen®). Blood samples were screened for *Plasmodium*, *Haemoproteus* and *Leucocytozoon* spp. using polymerase chain reaction (PCR).

We amplified a 515-bp fragment of cytochrome oxidase subunit-*b* gene (cyt *b*) for both *Plasmodium* spp. and *Haemoproteus* spp. This gene is commonly used for diagnostics of these parasites and has been shown to result in similar phylogenetic reconstructions as studies using nuclear genes (Hellgren *et al.* 2007a; Beadell *et al.* 2009; Valkiūnas *et al.* 2009). The following primers were used: L15183: 5′-GTGCAACYGTTATTACTAATTTATA-3′ and H15730: 5′-CATCCAATCCATAATAAAGCAT-3′ (Fallon *et al.* 2003; Szymanski & Lovette 2005). Reactions were carried out in a 25 μL reaction mixture containing 10–100 ng of genomic DNA (2 mL of template DNA), 10 mM Tris-HCl (pH8.3), 50 mM KCl, 3.0 mM MgCl, 0.4 mM of each dNTP, 0.4 mM of each primer, 5 μL of Q buffer, and 0.5 units *Taq* (Qiagen, 201203). We ran our amplifications as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles at 95 °C for 50 s, 53 °C annealing for 50 s, and 72 °C extension for 60 s, and then a final extension at 72 °C for 5 min.

To screen for the presence of *Leucocytozoon* spp., we used the same PCR reaction conditions as above in a nested PCR with the following primers as described in Hellgren *et al.* (2004); first set: HaemNR3: 5′-ATAGAAAGATAAGAAATACCATTC-3′ and HaemNFI: 5′-CATATATTAAGAGAAITATGGAGT-3′; second set: HaemFL: 5′-ATGGTGTTTTAGATA CTTACATT-3′ and HaemR2L: 5′-CATTATCTGGATGAGATAATGGIGCT-3′. The cycling profile conditions were: initial denaturation at 94 °C for 3 min, followed by 20 cycles 94 °C denature for 30 s, 50 °C annealing for 30 s, and 72 °C extension for 45 s and then a final extension at 72 °C for 10 min. We used 2 μL of the first PCR reaction as the template for the second PCR. The reaction conditions using the second primer set were identical to the first round and used the same cycling profile, but 35 cycles instead of 20. In some cases, we obtained two different sized PCR fragments, due to the first set of primers (Szöllosi *et al.* 2008). Thus, we used well-separating agarose gels, several positive controls, and molecular standards to facilitate separation of fragments, to correctly score positive infections. For *Leucocytozoon* spp., a subset of samples was sequenced for verification purposes. The hosts appear to harbour numerous multiple infections of these parasites, which would entail extensive efforts in cloning and sequencing. Hence, we report only prevalence data in this study.

PCR products were screened on 2% agarose gels, stained with ethidium bromide, and visualized with a UV light source. PCR products were purified for cycle sequence reactions using ExoSAP-IT (USB Corporation) following manufacturer's instructions. Bi-directional sequencing with dye-terminator fluorescent labelling was performed in an ABI Prism 3100 automated sequencer (Applied Biosystems, Inc.). We sequenced 515 base pairs for *Plasmodium* spp. and *Haemoproteus* spp. and 480 base pairs for *Leucocytozoon* spp., which were edited using Sequencher 4.8 (GeneCodes, Ann Arbor). Then sequences were distinguished by identifying their closest sequence matches in GenBank using the NCBI nucleotide Blast search. Unresolved sequences showing double peaks in the electropherograms were examined for putative multiple infections by cloning (TOPO-cloning kit, Invitrogen) and sequencing. Between 5 and 10 clones were sequenced from each

sample for which we observed a multiple infection. Unique sequences found multiple times in independent PCRs, either within the same individual or in several different individuals, were considered verified. In addition, parasite sequences differing by only one or two nucleotides were checked carefully against the original chromatogram. For verification purposes all unique lineages differing by one or two nucleotides were re-sequenced. With the initial 515 bp sequence of the mitochondrial cyt *b* gene we identified the individual parasite lineages; this size fragment has been found to accurately estimate the molecular diversity of the full cyt *b* gene for haemosporidian species identification (Hellgren *et al.* 2007a). In addition, sequences that differed by as few as one or two nucleotides were considered separate distinct lineages (Ricklefs & Fallon 2002). We conducted a Blast (Genbank's basic local alignment search tool) for each unique sequence (using the L15183/H15730 primer sets 515 bp) and identified matches for 12 out of the 26 lineages (Genbank accession number: DQ508376–DQ508395; Bonneaud *et al.* 2009). Using HaemNF/NR2 and HaemF/R2 primers, we found nine sequences to be the same; PV12L = pGRW9 (DQ060773), PV15L = pCYOL1 (EU770152), PV21L = pANLA1 (EU770151) and HV3L = h-ANLA1 (EU770153), PV23L = WA2 (EU810612), PV1L = WA16 (EU810619), PV3L = WA17 (EU810623), PV13L = WA4 (EU810658), HV1L = WAH14 (EU810739), and finally using the combine sequences we found two sequences to be the same; PV2L = Hap 50 (AF465550) and PV12L = Hap46 (DQ839085). All new sequences were deposited in GenBank (accession numbers FJ404695–FJ404720).

Phylogenetic analysis

For the purpose of increasing statistical power in the phylogenetic analyses, a second nested PCR protocol was used with the primers HaemNF/HaemNR2 and HaemF/HaemR2 as described in Waldenström *et al.* (2004). This was used for all distinct verified lineages of *Plasmodium* spp. and *Haemoproteus* spp. in order to increase the cyt *b* length to 750 bp. Phylogenetic analyses were implemented using maximum-likelihood (ML) techniques and sequence divergence algorithms were computed using PAUP* 4.0 (Swofford 2003). In addition, Bayesian analyses were used to generate a phylogeny of cyt *b* lineages. Sequence data was analysed using MrBayes version 3.1.2 (Huelsenbeck & Ronquist 2001) and implementing the model (GTR + G) obtained from MrModelTest. Two Markov chains were run simultaneously for 2.5 million generations with sampling every 100 generations for a total of 25 000 trees each, sampled from the posterior distribution. Those trees sampled prior to the runs reaching a split deviation frequency of 0.01 were discarded from the sample as the 'burn-in' period that accounted for 25% of the trees. The remaining trees were used to calculate the posterior probabilities of the individual clades. The support for the individual branches was estimated using ML bootstrap analyses running for 1000 replicates.

Sequence divergence values between *Haemoproteus* spp. and *Plasmodium* spp. lineages were analysed using Uncorrected-P distance. An additional distance analysis implementing a Jukes-Cantor model did not yield significantly different results.

Microscopic examination provided valuable information about co-infections of parasites belonging to the same and different genera in both host species (C. Loiseau, T. Iezhova, G. Valkiūnas & R.N.M. Sehgal, unpublished). We screened a subset of slides from 143 birds to verify PCR results and for identification of morphospecies. In cases where we did not obtain microscope results for a particular lineage due to light infections, we considered closely related lineages as one morphospecies when the genetic divergence within the clade was less than 5% (Hellgren *et al.* 2007a; Valkiūnas *et al.* 2009).

Statistical analyses

First, we conducted a species richness test between the two species of birds and between the paired sites using EcoSim 7.72 (Gotelli & Entsminger 2008) to provide rarefaction estimates. The program controls for differences in abundance, and was used to sample the common abundance level for 1000 iterations to create a mean diversity index, its variance, and a low and high bound for a 95% confidence interval.

Secondly, the association between the infection status and habitat type was tested using generalized linear models (Proc GENMOD with binomial distribution of errors and logit link function, SAS 1999). For each bird species and for each parasite genus, (*Plasmodium* spp., *Haemoproteus* spp. and *Leucocytozoon* spp.), in a first step, we investigated the relationship between the infection status (dependent, binary variable) and explanatory variables: the habitat (undisturbed vs. disturbed), the paired site (A–J), the season (dry vs. wet), and the year (2005 vs. 2007). In a second step, we performed additional analyses for four out of the six clades of parasites (Clade I, III, IV and V; Fig. 4) with the same explanatory variables (the two remaining clades of the six were represented by only one lineage).

In each case, the best model was selected by starting from a full model with all explanatory variables, and sequentially removing variables according to the Akaike information criterion (AIC). All analysis was conducted using the SAS software 9.1 (SAS, 1999).

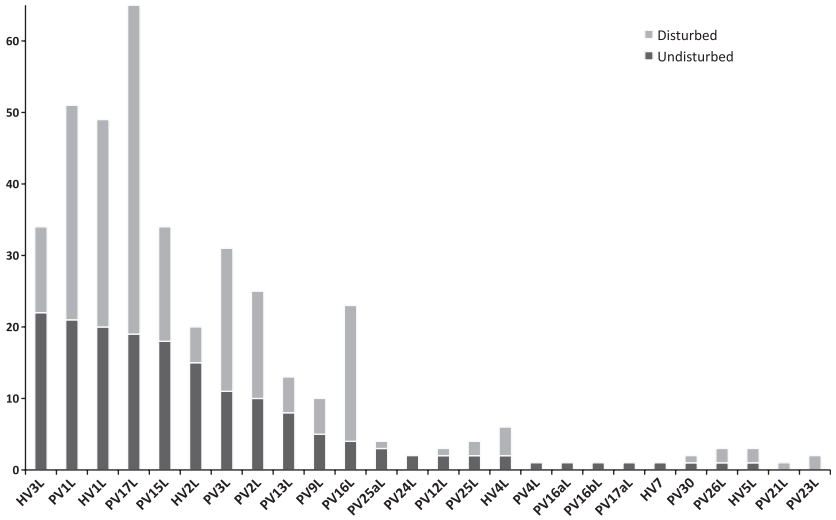

Fig. 2 Individual haemosporidian parasite lineages (*x*-axis) and numbers of recorded infections (*y*-axis) found at disturbed and undisturbed sites.

Results

Patterns of parasite diversity

Using PCR, we screened 851 birds of two species from two avian families (Table 1) and found 26 mitochondrial cyt *b* lineages; 20 *Plasmodium* spp. lineages (N_{total} = 277) and 6 *Haemoproteus* spp. lineages (N_{total} = 113; Supporting information Table S2, Fig. 2).

Differences were found in parasite diversity between the two different bird species. Overall, 13 *Plasmodium* spp. lineages and 5 *Haemoproteus* spp. lineages were found in *C. olivacea*. In *A. latirostris*, we found seven *Plasmodium* spp. lineages and only one *Haemoproteus* sp. lineage. The higher lineage richness in *C. olivacea* than in *A. latirostris* was confirmed by rarefaction analysis (Fig. 3).

We found one to three haemosporidian lineages (co-infections) in individuals of the two bird species. Only 2.4% of individuals (of both bird species) harboured more than one parasite lineage, and these multiply infected individuals were found in both disturbed and undisturbed sites.

The individual lineages were generally found to have a broad geographic distribution, with the same lineages being found at many of the paired sites. For example, lineages PV1L and HV1L were found at 14 and 16 of the sites respectively. The EcoSim rarefaction species richness test found two paired sites to exhibit higher than average diversity: both paired sites E and H (Fig. 1) showed the highest species richness values at 15.7 and 15.5 respectively (overall lineage richness varied from 4.5 to 15.7) even when accounting for sample size.

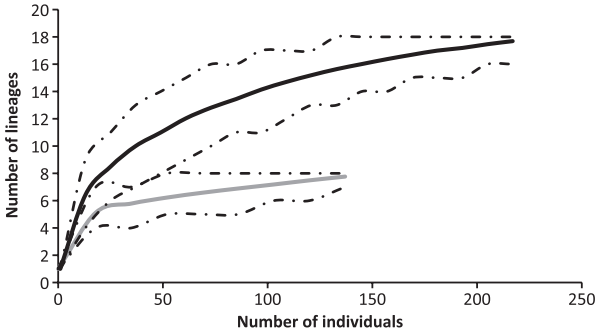

Fig. 3 Haemosporidian parasite lineage richness curves for *Cyanomitra olivacea* and *Andropadus latirostris*. Dashed lines represent the 95% high and low confidence values.

Phylogenetic relationships

Phylogenetic analysis revealed two highly supported monophyletic clades for *Plasmodium* spp. Genetic distances within lineages of the *A. latirostris* clade ranged from 0.1% to 5.7% and within the *C. olivacea* clade from 0.1% to 7.9% (Fig. 4). These clades showed marked host-species fidelity. However, from the *A. latirostris* clade, PV1L was found once in *C. olivacea*, but PV25L + PV25aL (which differ by only 1 nucleotide) were found in seven individuals of *C. olivacea*. Interestingly, all infections of PV25 + PV25aL were only found in multiply infected samples. From the *C. olivacea* clade, PV15L was found in one *A. latirostris*. Analysis revealed a single clade for *Haemoproteus* with HV1L, HV2L, HV4L and HV5L infecting only *C. olivacea* (with a within clade genetic distance between 0.2% and 2.5%) and *Haemoproteus vacuolatus* (HV3L) infecting only *A. latirostris*. The genetic distance for *Plasmodium* spp.

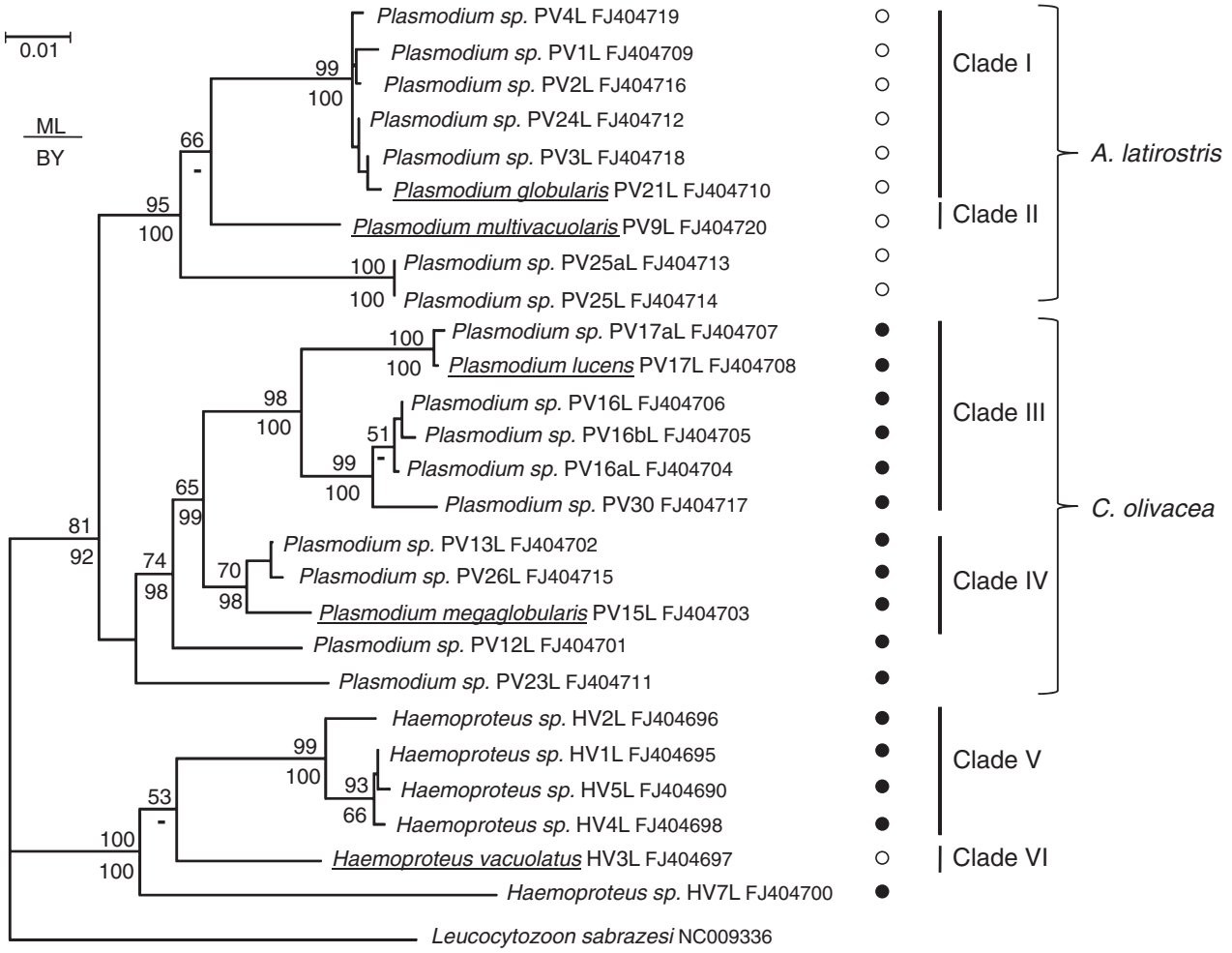

Fig. 4 Phylogram of *Haemoproteus* spp. and *Plasmodium* spp. lineages in *Andropadus latirostris* (white circles) and *Cyanomitra olivacea* (black circles). Phylogenetic relationships of the 26 haemosporidian parasite lineages found in two species of birds, based on cyt *b* sequences. *Leucocytozoon sabrazesi* was used as the outgroup. Codes of lineages and GenBank accession numbers of all sequences are indicated after species names. Numbers located on the top of the branches indicate ML bootstrap support (1000 replications, only values above 50% are shown) and below are from Bayesian probability values. The brackets indicate the two clades of *Plasmodium* lineages. Vertical bars correspond to clades of closely related lineages that belong, or likely belong, to the same morphospecies of parasites.

between the *A. latirostris* and the *C. olivacea* lineages varied from 6.4% to 9.9%, and between *Plasmodium* spp. and *Haemoproteus* spp. lineages, 8.8–12.2%.

Within the major *Plasmodium* and *Haemoproteus* clades, we assigned morphospecies nomenclature to smaller cladistic groupings when microscopy data were available (clades I to VI; Fig. 4). For the *Plasmodium* spp., we found four distinct clades representing probable morphospecies with pairwise genetic distances within them between 0% and 3.7%: Clade I – *P. globularis*, Clade II – *P. multivacuolaris*, Clade III – *P. lucens*, Clade IV – *P. megaglobularis*. For *Haemoproteus* spp., we found two distinct clades, which correspond to two probable morphospecies; (Clade V – *Haemoproteus* sp. (undescribed), Clade VI – *H. vacuolatus*). Within-clade genetic distances ranged from 0.2% to 2.5%.

Habitat effect on prevalence

First, in *C. olivacea* ($N = 440$), the prevalence of haemosporidian infections was 31% in disturbed habitat vs. 23% in undisturbed habitat. Prevalence of *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. was 18%, 33% and 48% respectively.

Leucocytozoon spp. prevalence was significantly higher in undisturbed than disturbed habitat (undisturbed = 59.0%; disturbed = 40.5%, $\chi^2 = 14.28$, $P < 0.0017$). We found a season effect with a higher prevalence in the

dry season ($\chi^2 = 12.25$, $P < 0.0005$), a year effect, prevalence was higher in 2007 ($\chi^2 = 17.95$, $P < 0.0001$), as well as a paired site effect ($\chi^2 = 9.6$, $P < 0.0001$).

Haemoproteus spp. prevalence differed significantly between habitat types (disturbed = 14.6%, undisturbed = 22.9%; $\chi^2 = 4.77$, $P < 0.0290$). Clade V, which includes four out of the five *Haemoproteus* lineages, exhibited the same pattern ($\chi^2 = 4.15$, $P = 0.0418$). For *Plasmodium* spp. we did not see any significance for habitat effect (disturbed = 33.6%, undisturbed = 33.1%; $\chi^2 = 0.02$, $P = 0.8969$) at the genus level of parasites, but upon closer examination of the clades, we did in fact find a significant habitat effect. Indeed, two of the clades showed a significant but opposite effect, with a higher prevalence in undisturbed than disturbed habitat for the clade IV (*P. megaglobularis*) ($\chi^2 = 4.59$, $P = 0.0322$; Fig. 5) whereas clade III (*P. lucens*) exhibited the inverse, with a higher prevalence in disturbed than undisturbed habitat ($\chi^2 = 5.32$, $P = 0.0211$). No effects of season, year or paired site were found on the prevalence of either *Plasmodium* or *Haemoproteus* infections.

Second, in *A. latirostris* ($N = 411$), the prevalence of haemosporidian infection was 20% in disturbed habitat vs. 17% in undisturbed habitat. Prevalence of *Haemoproteus* spp., *Plasmodium* spp. and *Leucocytozoon* spp. was 8%, 29% and 36% respectively.

Similar to *C. olivacea*, *Leucocytozoon* spp. prevalence was significantly higher in undisturbed than disturbed habitat (disturbed = 29.2%, undisturbed = 43.1%; $\chi^2 = 5.49$, $P < 0.019$) and we found a paired site effect ($\chi^2 = 9.57$, $P < 0.0001$).

Only one *Haemoproteus* species, *H. vacuolatus*, was found in *A. latirostris*, and we did find a marginal habitat effect on the prevalence, with higher prevalence, again, in undisturbed habitat (disturbed = 5.8%, undisturbed = 10.9%; $\chi^2 = 3.65$, $P < 0.056$), whereas *Plasmodium* spp. prevalence was significantly higher in disturbed than undisturbed habitat (disturbed = 34.1%, undisturbed = 24.3%; $\chi^2 = 4.91$, $P < 0.026$). Clade I showed the same pattern, with a significantly higher prevalence in disturbed than undisturbed ($\chi^2 = 5.62$, $P = 0.0178$). We found a season effect on both *Plasmodium* spp. and *Haemoproteus* spp. prevalence, with a higher prevalence during the wet season ($\chi^2 = 13.8$, $P = 0.0002$ and $\chi^2 = 4.04$, $P = 0.044$).

Discussion

As humans continue to alter habitats, the implications and consequences for the transmission and distribution of infectious diseases are paramount. Thus, it has become essential to understand and measure how parasites and their hosts respond to habitat changes. Here, we demonstrated that anthropogenic changes correlate with variation in the (i) diversity, (ii) prevalence; and (iii) distribution of haemosporidian parasites in two widespread African rainforest bird species. We found that different bird species can exhibit opposing trends in parasite prevalence in disturbed and undisturbed habitats. In addition, we found a strong host effect on the prevalence of haemosporidian parasites. Indeed, we found *C. olivacea* not only exhibited higher prevalences for parasites of all three genera, but also possessed a

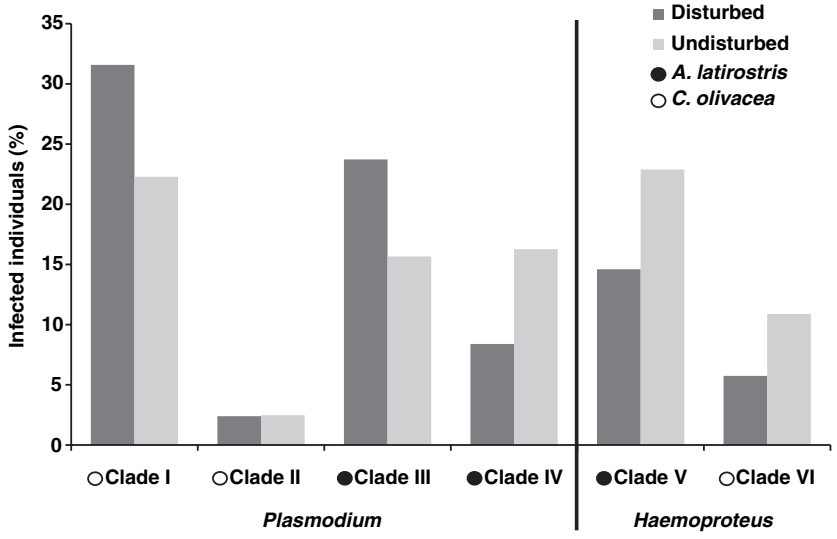

Fig. 5 Haemosporidian parasite prevalence by lineages of different clades, each likely representing a distinct morphospecies. Percent prevalence for each clade or morphospecies recorded from all disturbed (grey bar) and undisturbed (light grey bar) study sites. The *y*-axis is the prevalence of infection in percentage.

significantly higher richness of parasite morphospecies and associated lineages than *A. latirostris*. Our results show that parasite morphospecies can differ in their distributions with regard to habitat type. Finally, we discovered marked geographic differences in parasite lineage diversity among paired sites.

Parasite prevalence in altered habitats

Comparing sites on a reduced spatial scale, we eliminate many of the confounding environmental variables (such as rainfall, temperature) that could affect parasite transmission. Paired sites were chosen so that they would share these abiotic factors and thus reveal differences solely caused by human-induced habitat changes. Indeed, when comparing prevalences of parasites on a large scale (i.e. between all disturbed and undisturbed sites), we did discover significant differences. Overall, we found that *Haemoproteus* spp. and *Leucocytozoon* spp. prevalences were significantly higher in undisturbed habitats for both hosts. These results are similar to the findings of Bonneaud *et al.* (2009), which found a higher prevalence of *Plasmodium* spp. in undisturbed forests of Cameroon in *Andropadus virens*, *A. latirostris* and *C. obscura* (=*C. olivacea*). Those findings were conducted at just four sites, from disparate regions of Cameroon, and provided the impetus for this more comprehensive work. In contrast to the findings by Bonneaud *et al.* (2009), for *A. latirostris*, when we analysed the *Plasmodium* spp. prevalence, we found a significantly higher prevalence in disturbed habitat, corroborating results studying human malaria (Vittor *et al.* 2006).

With *C. olivacea*, we did not find an effect for habitat type on the prevalence of combined *Plasmodium* species and their lineages. However, examining the individual clades, we found opposing effects of habitat type on the two *Plasmodium* spp. clades (III and IV) that represent the majority (94%) of *Plasmodium* spp. infections in *C. olivacea*. Lineages of these clades most likely represent two distinct morphospecies [*P. megaglobularis* and *P. lucens* respectively, both belonging to avian malaria parasites of the subgenus *Novyella* (see Valkiūnas *et al.* 2008b, 2009)]. It was found that lineages representing *P. megaglobularis*, exhibited a higher prevalence in undisturbed habitat, and lineages of *P. lucens* exhibited higher prevalence in disturbed habitats. These results demonstrate the utility of a method of summarizing prevalence data across closely related lineages of the same morphospecies of parasites. These methods help elucidate the complex distribution of parasites, and stress the importance of studying groups of related lineages of parasites representing separate morphospecies. This study also emphasizes that summarizing distribution data of all lineages belonging to one haemosporidian genus might mask some patterns of infection distribution, and should be used with caution in studies of ecological and evolutionary biology.

There are several hypotheses that can account for these contrasting effects of habitat on parasite prevalence. This may be a clear example of a parasite–host association being driven by vector specificity, with the individual species of vectors competing with variable success in these changing environments. This study suggests that distinct parasite morphospecies may be transmitted by species of mosquitoes that are affected differently by changes in habitat. This is not surprising since habitat degradation has been shown to have variable impacts on the diversity of mosquito species (Beck *et al.* 1994; Vittor *et al.* 2006; Reiter & LaPointe 2007; Vanwambeke *et al.* 2007; Yasuoka & Levins 2007). Thus, one hypothesis is that the vector species that transmit morphospecies that possess a higher prevalence in disturbed areas, exhibit increased fitness in these habitats, and are therefore found in greater prevalence. The species of vectors are still unknown for all species of haemosporidians mentioned in this study, as is true for the great majority of avian haemosporidian species in Africa (Valkiūnas *et al.* 2009). Future research identifying and studying the abundance of vectors at these sites will help clarify these results.

In addition, there is a possibility that changes or shifts in vector feeding habits coincide with environmental changes. Vectors could change from primarily zoophilic to primarily anthrophilic with ecological disruption, resulting in a decrease in parasite prevalence in the original hosts. In fact, studies have found a substantial increase in human feeding rates by *Anopheles* mosquitoes in areas that have undergone deforestation (Vittor *et al.* 2006). In addition, other studies have reported feeding shifts by mosquitoes from birds to humans when the numbers of their preferential hosts are reduced (Kilpatrick *et al.* 2006). Recent studies have shown that mosquitoes may not restrict the access of parasites to birds but instead carry and transmit a diverse array of parasite lineages (Gager *et al.* 2008; Ishtiaq *et al.* 2008). Alternatively, there may be an increase in the density of alternative hosts in disturbed sites, which are not competent, or less susceptible to infection. A consequence may be a decrease in the prevalence, and in the risk of infection at disturbed sites through a 'dilution effect' (Ostfeld & Keesing 2000; Schmidt & Ostfeld 2001).

Similar to results of previous studies (Ricklefs & Fallon 2002; Hellgren *et al.* 2007b), parasite lineages showed marked host fidelity to the two host species. Here, we found 26 parasite lineages, but only three were shared between the two hosts. These findings add

to studies that report the occurrence of host switching between birds of different families (Ricklefs *et al.* 2004; Križanauskienė *et al.* 2006). Considering the extensive parasite diversity and high prevalence found, we would expect multiple infections to be more common (Mayxay *et al.* 2004). In this study, however, fewer than 3% of individuals harboured more than one parasite lineage. This implies again a high degree of host–vector–parasite specificity. Interestingly, the lineages PV25 + PV25aL were only found in mixed infections, suggesting that other lineages may somehow facilitate their infection success. However, the relatively low rate of co-infection may be due to the choice of primers, and that PCR underestimates the occurrence of mixed infections (Valkiūnas *et al.* 2006). This demonstrates the necessity of both microscopy and PCR for the screening of parasites in studies of haemosporidian biodiversity (C. Loiseau, T. Iezhova, G. Valkiūnas & R.N.M. Sehgal, unpublished; Valkiūnas *et al.* 2008b, 2009).

Host effects on parasite transmission

Since prevalences of parasites of all three genera were markedly higher in *C. olivacea* than in *A. latirostris*, these bird species may have underlying differences in their evolutionary strategies in immune system investment. Indeed, the effects of parasites on hosts have been shown to vary in relation to the genotype of the host (Westerdahl *et al.* 2005; Bonneaud *et al.* 2006; Loiseau *et al.* 2008). Differences in prevalence between the two species might be due to the trade-offs that occur between investment in their immune system and reproduction (Apanius *et al.* 1994; Sheldon & Verhulst 1996; Lochmiller & Deerenberg 2000; Norris & Evans 2000; Tomas *et al.* 2007). Alternatively, these two species may differ in their attractiveness to vectors, as has been observed in some other bird species (Hamer *et al.* 2009).

Two of the seven observed haemosporidian lineages in *A. latirostris* were newly described. The other five lineages were found in a previous study in Cameroon (Bonneaud *et al.* 2009) and three of them were found in more than one species, but always in the same bird family (i.e. Pycnonotidae). In *C. olivacea*, of the 13 lineages reported here, 8 are new. The remaining five were found previously (Bonneaud *et al.* 2009), and two of them were found in two different bird families. As compared to previous studies, we found considerably more parasite lineages in *C. olivacea* than *A. latirostris*. Indeed, the species richness index (Fig. 3) clearly shows a higher overall diversity of parasites for *C. olivacea* that is independent of sample size. Why are certain hosts subject to a higher diversity of parasites in the same habitat? Similar to explanations regarding differences in prevalence, we expect the composition of the parasite community to be determined by the variation in selection pressures imposed by the host's individual immunity on different parasite lineages. Another explanation may be that individual parasite lineages that compete for limited resources may be more or less competitive and successful in different hosts (i.e. generalist vs. specialist parasites; Woolhouse *et al.* 2001). Alternatively, exposure to multiple vectors or multiply infected vectors could result in increased parasite species richness in certain hosts (Paul *et al.* 2002). Feeding specialization of vectors (their preference to *C. olivacea*), or behaviours of the hosts also might contribute to this result (Malmqvist *et al.* 2004; Hellgren *et al.* 2008; Hamer *et al.* 2009).

Parasite distribution

The geographical distribution of genetically distinct avian haemosporidian parasites has been studied widely (Bensch *et al.* 2000; Ricklefs & Fallon 2002; Waldenström *et al.* 2002; Wood *et al.* 2007; Beadell *et al.* 2009) but still warrants further investigation with regard to patterns of overall biodiversity, especially in the tropics, and in relation to deforestation. On the local scale, Wood *et al.* (2007) discovered several complex patterns of prevalence with respect to landscape features. Such heterogeneity in parasite infection at this scale provides information on how lineages may be transmitted within a small area, but does not explain the unexpected widespread transmission of certain parasites. In this study, we found that most lineages had a broad geographic distribution. For example, seven *Plasmodium* and four *Haemoproteus* lineages described here were also found in Ghana, across the Dahomey Gap (C. Loiseau, T. Iezhova, G. Valkiūnas & R.N.M. Sehgal, unpublished), where evolutionary relationships of bird species suggest a split of approximately 2 million years (Smith *et al.* 2001). Moreover, some sites exhibited higher parasite diversity than others. For example, we found that two paired sites E and H had the highest mean richness values. A possible explanation may be the presence of important watershed areas within close proximity to these sites (within 1 km). Paired site E is located adjacent to the Rio-del-Rey estuarine area and site H, near the Douala-Edea Faunal Reserve. These two areas are considered important coastal zone mangrove estuarine systems of Cameroon. These areas are well known to support a rich aquatic fauna as well as a high diversity of bird species that are know to gather in large numbers (Thomas 1995). A high number of microhabitats for the development of vectors might contribute to higher vector diversity resulting in greater parasite diversity. It seems probable that areas of generally recognized high biodiversity also appear to

maintain high parasite diversity, if abiotic and other factors are favourable for transmission.

Many earlier studies have focused on the evolution and ecology of parasites in multiple bird species. Our study provides data on the effects of deforestation on relatively large sample sizes of two widespread species of African rainforest birds. Our work also underscores the importance of studying morphospecies of parasites, and not only parasite lineages, since distinct morphospecies, even those that are close genetically and taxonomically (Valkiūnas *et al.* 2008b, 2009), clearly differ ecologically. We showed that habitat changes can affect host–parasite systems. These systems are complex, with many biotic and abiotic variables and the work presented here raises many questions regarding the mechanisms underlying how deforestation results in changes in parasite prevalence. It is becoming increasingly clear that the insect vectors' response to environmental change can be a major factor regulating haemosporidian parasite distribution and prevalence. For this reason, it is imperative to initiate concerted sampling efforts for the dipteran insect vectors, including investigations on the development of parasites in the vectors. It is also clear that different bird species can respond differently to changes in parasite communities, thus stressing the importance of immune system research. In all, with the rapid decline of forests in Africa, this research accentuates the sensitivity of these ecosystems to change, and emphasizes the importance of conservation in the maintenance of complex parasite diversity as an integral component of all biodiversity.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Disturbed and undisturbed paired study sites with Latitude and Longitude coordinates. Total sample numbers from each site for both species Cyol: *Cyanomitra olivacea* and Anla: *Andropadus latirostris*

Table S2 Total number of individual haemosporidian parasite lineages and numbers of recorded infections at disturbed and undisturbed sites

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