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SPATIAL VARIATION OF HAEMOSPORIDIAN PARASITE INFECTION IN AFRICAN RAINFOREST BIRD SPECIES

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ABSTRACT: Spatial heterogeneity influences the distribution, prevalence, and diversity of haemosporidian parasites. Previous studies have found complex patterns of prevalence with respect to habitat characteristics and parasite genotype, and their interactions, but there is little information regarding how parasitemia intensity and the prevalence of co-infections may vary in space. Here, using both molecular methods and microscopy, we report an analysis of the variation of parasitemia intensity and co-infections of avian haemosporidian parasites (*Plasmodium* and *Haemoproteus* species) in 2 common African birds species, the yellow-whiskered greenbul (*Andropadus latirostris*) and the olive sunbird (*Cyanomitra olivacea*), at 3 sites with distinct habitat characteristics in Ghana. First, we found an interaction between the site and host species for the prevalence of *Plasmodium* spp. and *Haemoproteus* spp. For the olive sunbird, the prevalence of *Plasmodium* spp., as well as the number of individuals with co-infections, varied significantly among the sites, but these measures remained constant for the yellow-whiskered greenbul. In addition, yellow-whiskered greenbuls infected with *Haemoproteus* spp. were found only at 1 site. Furthermore, for both bird species, the parasitemia intensity of *Plasmodium* spp. varied significantly among the 3 sites, but with opposing trends. These results suggest that spatial heterogeneity differently affects haemosporidian infection parameters in these vertebrate-hosts. Environmental conditions here can either favor or reduce parasite infection. We discuss the implications of these discrepancies for conservation and ecological studies of infectious diseases in natural populations.

Spatial heterogeneity affects community structure and ecological processes, including host–parasite interactions. Transmission of infectious disease is a result of complex interactions between abiotic factors and biotic components. First, environmental conditions can strongly impact parasite species diversity and abundance, either favoring or limiting parasite numbers and, thereby, affecting the prevalence of host infection. Several studies have shown correlated changes in vector dynamics (Walsh et al., 1993; Poncon et al., 2007), transmission rates (Kutz et al., 2005; Greer and Collins, 2008), parasite host ranges, and parasite virulence (Lebarbenchon et al., 2008) following climatic or habitat changes. How such instabilities in host–parasite interactions will affect evolutionary processes remains difficult to predict, as each host–parasite system probably responds individually and independently. Clearly, extrinsic factors, but also intrinsic factors associated with the hosts, such as genotypic resistance (Westerdahl et al., 2005; Bonneaud et al., 2006; Loiseau et al., 2008), behavior, age, or sex (McCurdy et al., 1998; Ots and Horak, 1998) can also be responsible for infection prevalence.

Previous investigations have established that infections by avian haemosporidian parasites (Haemosporida) can vary in space (Merila et al., 1995; Sol et al., 2000; Bensch and Åkesson, 2003; Gibb et al., 2005; Wood et al., 2007; Svensson and Ricklefs, 2009). These parasites are almost worldwide in distribution and possess a wide range of potential hosts. Blood-sucking dipteran insects are vectors of haemosporidians, i.e., mosquitoes transmit *Plasmodium* spp., and biting midges and louse-flies transmit species of *Haemoproteus* (Valkiūnas, 2005). The role of vectors is one of the important components in haemosporidian transmission, as vectors clearly determine the access of blood parasites to vertebrate hosts in ecological time (Sol et al., 2000; Gager et al., 2008; Hellgren et al., 2008; Ishtiaq et al., 2008; Kimura et al., 2010). Abiotic factors, such as climatic conditions and habitat characteristics, also play a pivotal role because they may contribute to changes in both vector and host diversity–abundance, resulting in changes in parasite transmission dynamics along with possible modifications in parasite virulence, local host–parasite adaptation, and consequent changes in host reproductive success.

Effects of landscape characteristics on the prevalence of avian species of *Plasmodium* and *Haemoproteus* have been investigated at different scales (Wood et al., 2007; Bonneaud et al., 2009; Chasar et al., 2009). Wood et al. (2007) examined variation in avian malaria infection, with respect to landscape and host factors, at a small scale, i.e., a single woodland population of blue tits, *Cyanistes caeruleus*. They found that variation in prevalence between woodland sections varied between lineages, indicating that different lineages had different spatial distributions, with an increased prevalence for 2 lineages that were in the proximity of a large body of water. At a large scale, in Cameroon, Bonneaud et al. (2009) and Chasar et al. (2009) demonstrated that anthropogenic habitat change, i.e., deforestation, can affect host–parasite systems in terms of diversity and distribution, resulting in opposing trends of haemosporidian prevalence in wild bird populations. In evaluating transmission capabilities, prevalence is a parameter that we can estimate relatively easily. However, few studies on avian haemosporidian parasites have investigated parasitemia intensity, as well as co-infection occurrence, in relation to spatial heterogeneity: 2 important components reflecting parasite fitness. Intensity of parasitemia can be used as a relative proxy for virulence i.e., the damage done to the host, because it reflects parasite reproductive success and may be related to parasite-induced morbidity and mortality (Mackinnon and Read, 2004; Palinauskas et al., 2008). In addition, infection of hosts by multiple species occurs in many host–parasite systems (Read and Taylor, 2001) and may either increase (Mosquera and Adler, 1998; Taylor et al., 1998) or decrease (Taylor et al., 2002; de Roode et al., 2003) parasite virulence.

Therefore, here, we examined the impact of spatial heterogeneity on parasitemia intensity and co-infections of haemospor-

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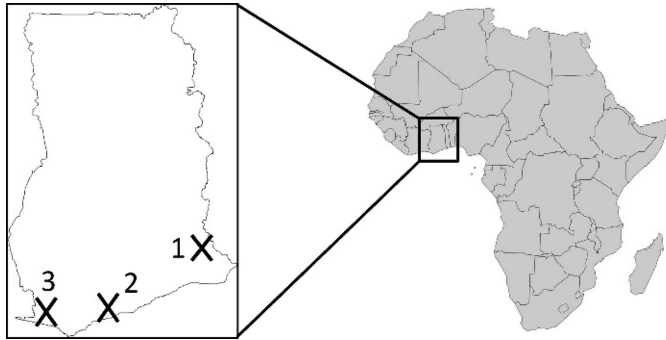


FIGURE 1. Map showing location of three study sites (1–3) in Ghana (black crosses represent the collection sites).

idian parasites in 2 common African birds, the yellow-whiskered greenbul (*Andropadus latirostris*) and the olive sunbird (*Cyanomitra olivacea*) at 3 sites in Ghana. These 3 sites were chosen due to their known environmental characteristics in terms of habitat, i.e., levels of deforestation and altitude as well as climatic conditions, i.e., seasonal rainfall. Controlling for the season, which could influence variation in parasite prevalence (Altizer et al., 2006; Cosgrove et al., 2008), and also for avian species allowed us to focus on spatial heterogeneity effects in host–parasite interactions. We predicted that both environmental characteristics, and host species and their interactions, should affect patterns of parasitemia intensity and co-infections, giving rise to different assemblages of parasite lineages at each site. Because recent studies have exposed the limitations of molecular methods in diagnostics of co-infections in wildlife (Valkiūnas et al., 2006; Valkiunas, Iezhova et al., 2008; Martínez et al., 2009), we used PCR with sequencing and microscopic examination of blood films to test how parasitemia and co-infections, along with prevalence, can vary between bird species and sites in a tropical rainforest ecosystem.

MATERIALS AND METHODS

Sample sites and field methods

Field work took place in July 2007 at 3 sites in Ghana (Fig. 1): Agumatsa (Site 1: 07°01.758′N, 00°33.490′E; altitude 269 m), Abrafo (Site 2: 05°21.171′N, 01°23.406′E; altitude 170 m), and Nkwanta (Site 3: 05°16.912′N, 02°38.495′E; altitude 85 m). Agumatsa is characterized by a drier climate and a high level of deforestation, with only 27% of tree cover remaining. Nkwanta, in Ankasa National Park, although a secondary forest, has much lower levels of disturbance, with a 64% tree cover. Abrafo, on the edge of Kakum National park, is considered intermediate in terms of rainfall and deforestation in comparison to the other 2 sites, with 41% of tree cover remaining. Each site differs in bioclimatic measures (temperature and rainfall) and habitat characteristics (Table I). Spatial distribution of vegetation density is given by the tree cover layer as well as by the Normalized Difference Vegetation Index (NDVI), based on monthly files from 2001 MODIS data (Hansen et al., 2002), whereas surface moisture and roughness, i.e., forest structure, is given by the annual mean radar backscatter (from QuikSCAT satellite; http://manati.orbit.nesdis.noaa.gov/cgi-bin/qscat_storm.pl).

TABLE II. Sample size and prevalence (%; combining microscopy and PCR data) of *Plasmodium* spp. and *Haemoproteus* spp. are given by bird species and study site.

Bird species		Site	*n*	*Plasmodium*	*Haemoproteus*
Andropadus latirostris	(1)	Agumatsa	45	27.0	0
	(2)	Abrafo	24	25.0	0
	(3)	Nkwanta	40	22.5	25.0
Cyanomitrae olivacea	(1)	Agumatsa	33	42.4	42.4
	(2)	Abrafo	30	60.0	43.3
	(3)	Nkwanta	41	82.9	48.8

Each site was sampled for 4 consecutive days. On average, 16 mist nets (12 m, 30 × 30-mm mesh) were erected to capture birds between 0530 and 1400 hr. Blood samples were collected from the brachial vein and stored in lysis buffer (10 mM Tris-HCL pH 8.0, 100 mM EDTA, 2% SDS). We captured 213 birds of the 2 most-common species (Table II). In total, 109 yellow-whiskered greenbul (Pycnonotidae) were sampled (Table II) at Agumatsa (site 1) n = 45, Abrafo (site 2) n = 24, and Nkwanta (site 3) n = 40; we also sampled 104 olive sunbirds (Nectariniidae): (site 1) n = 33, (site 2) n = 30, and (site 3) n = 41. All birds were banded and body mass was determined; olive sunbirds were sexed based on the presence of sexually dimorphic pectoral feather tufts. The 2 target species are non-migratory (Cheke et al., 2001). The olive sunbird is known to be mainly sedentary and prefers to forage in the lower strata (0–6 m), with an insectivorous and a nectivorous diet (Cheke et al., 2001). The yellow-whiskered greenbuls are omnivorous; they are polygamus and non-territorial, with some evidence of cooperative breeding. Both of these species can be commonly found in primary and secondary forests as well as in agroforestry systems (Bobo, 2007).

Parasite screening using PCR

DNA was extracted from whole blood following a DNeasy kit protocol (Qiagen, Valencia, California). Success of each DNA extraction was verified with primers that amplify the brain-derived neurotrophic factor (BDNF) gene (Sehgal and Lovette, 2003).

We used 2 PCR methods for *Plasmodium* and *Haemoproteus* spp. detection. One amplifies a fragment of the cytochrome b gene (510 bp) of the mtDNA with the following primers: L15183: 5′-GTG CAA CYG TTA TTA CTA ATT TAT A-3′ and H15730: 5′-CAT CCA ATC CAT AAT AAA GCA T-3′ (Fallon et al., 2003; Szymanski and Lovette, 2005). The cycling profile consisted of an initial denaturation at 94 C for 3 min, followed by 35 cycles of 94 C for 50 sec, 53 C annealing for 50 sec, and 72 C extension for 60 sec, and then a final extension at 72 C for 5 min. We also used a nested PCR to amplify a fragment of the cytochrome b (524 bp) of both *Plasmodium* spp. and *Haemoproteus* spp. lineages found from infected birds with the primers HAEMF/HAEMR2 – HAEMNF/HAEMNR2 following Waldenström et al. (2004). By utilizing both

TABLE I. Climatic and habitat variables by study sites. Annual temperature (A), as well as maximum temperature of warmest (W) and minimum temperature of coldest (C) month, are given in degrees Celsius (C). Annual (A), wettest- (W), and driest- (D) quarter precipitation is given in mm. The percentage of tree cover from MODIS data (10-km buffer around the site of capture), annual maximum NDVI (leaf area index; based on monthly files from MODIS data; 1-km resolution) and Qscat Mean (annual mean radar backscatter in Dezibel) are also reported.

		Temperature			Precipitation					
	Site	A	W	C	A	W	D	Tree cover	NDVI	QSCAT
(1)	Agumatsa	25.9	33.6	19.8	1,533	541	117	27	8,284	−8.124
(2)	Abrafo	25.3	30.7	20.5	1,439	620	137	41	8,828	−7.193
(3)	Nkwanta	26.4	31.9	21.8	1,969	902	194	64	9,438	−6.735

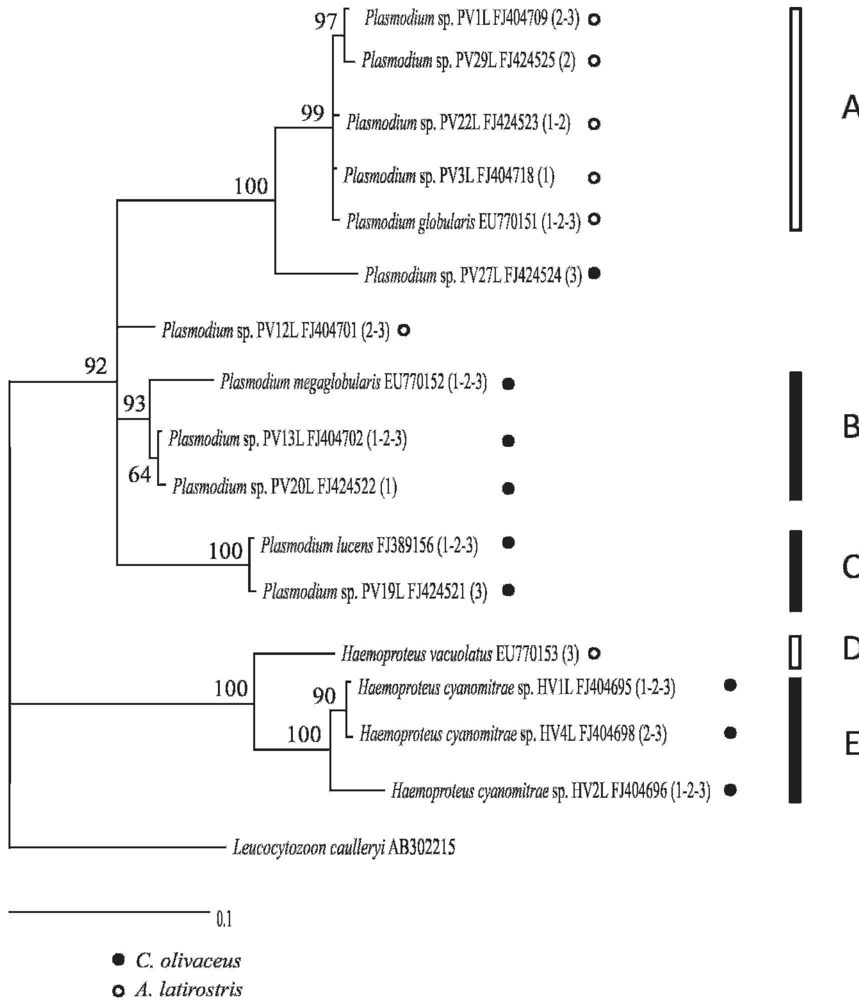


FIGURE 2. Phylogenetic relationships among lineages of 16 haemosporidian parasites. *Leucocytozoon caulleryi* was used as outgroup. GenBank accession numbers of all sequences are indicated. Numbers along branches correspond to node support from Bayesian analysis. The site number where the lineages were found, as well as the host species, is reported. Each vertical bar represents a clade (A to E) corresponding to one morphospecies: A = *Plasmodium globularis*, B = *Plasmodium megaglobularis*, C = *Plasmodium lucens*, D = *Haemoproteus vacuolatus*, E = *Haemoproteus cyanomitrae*.

PCR protocols in combination, we were able to obtain a fragment of the cyt b (750 bp). We compared the lineages with all sequences from blood parasites already deposited in Genbank.

Positive and negative controls were used, i.e., positive controls were birds with known infections, as indicated by microscopy results; the negative controls used purified water in place of a DNA template, or else samples that were consistently void of parasites as confirmed by microscopy and PCR. The PCR products were run out on a 2% agarose gel using 1× TBE and visualized by an ethidium bromide stain under ultraviolet light.

PCR products from birds infected with *Plasmodium* and *Haemoproteus* spp. were purified using ExoSap (following manufacture's instructions; USB Corporation, Cleveland, Ohio). We identified lineages by sequencing the fragments (BigDye [R] version 1.1 sequencing kit, Applied Biosystems, Foster City, California) on an ABI PRISM 3100 (TM) automated sequencer (Applied Biosystems). All unique sequences were verified by a second sequencing. Sequences are deposited in GenBank (accession numbers in Fig. 2).

Parasite screening using microscopy

From each bird, 2 or 3 blood films were prepared on glass slides. Blood films were air-dried within 5–15 sec after their preparation. We used a battery-operated fan to aid in the drying of blood films. Smears were fixed in absolute methanol for 1 min on the day of their preparation. Fixed smears were air-dried and packed into paper bands so that they did not touch each other. 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 solution of a commercially purchased stock solution of Giemsa, pH 7.0–7.2, at 18–20 C for 1 hr. All blood films were stained between 25 and 30 days after fixation. Details of preparation and staining of blood films are described by Valkiūnas (2005).

One blood film from each infected bird was examined using an Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera (Olympus) and imaging software AnalySIS FIVE (Olympus Soft Imaging Solutions GmBH, Münster, Germany). Only good-quality slides, i.e., without any features of lysis of cells and with well-stained blood cells and parasites, were used for microscopic examination. Approximately 100 fields were viewed at low magnification (×400), and then at least 100 fields were studied at high magnification (×1,000) as described by Valkiūnas, Iezhova, Križanauskienė et al. (2008). Each sample was examined for 20–25 min. In total, the approximate number of screened red blood cells was 5×10^5 in each blood film. Intensity of infection was estimated as a percentage by counting the number of parasites per 10,000 erythrocytes examined, as recommended by Godfrey et al. (1987). We log transformed all estimates of parasitemia intensity before statistical analyses.

Phylogenetic analyses

A phylogenetic tree was constructed using 12 mitochondrial cytochrome b sequences of avian *Plasmodium* spp. and 4 of *Haemoproteus* spp. from our survey. Of these, 5 lineages were recently described as new species (*Plasmodium globularis*, *Plasmodium megaglobularis*, *Plasmodium lucens*, *Haemoproteus vacuolatus*, *Haemoproteus cyanomitrae*; Valkiūnas, Iezhova, Loiseau et al., 2008; Iezhova et al., 2009; Valkiūnas et al. 2009). Morphospecies were not identified for all the recorded lineages (Fig. 2) because of a low intensity of parasitemia, an absence of all stages of

TABLE III. Prevalence (%) of *Plasmodium* spp. and *Haemoproteus* spp. with both PCR and microscopy methods.

Species	*Plasmodium* spp.		*Haemoproteus* spp.	
	PCR	Microscopy	PCR	Microscopy
Andropadus latirostris	21.1	17.4	9.2	9.2
Cyanomitrae olivacea	23.1	62.5	38.4	33.6

parasites necessary for species identification, or because of frequent co-infections.

The sequences were aligned using Sequencher 4.8 (GeneCodes, Ann Arbor, Michigan). All individual sequences were grouped into a consensus that was 750 bp long, with *Leucocytozoon caulleryi* (GenBank AB302215) used as an outgroup. We used Bayesian analysis to construct a phylogeny of parasite cytochrome b lineages. We first determined the model of sequence evolution that best fit the data using MrModeltest (Nylander, 2004). Bayesian analysis of the sequence data was then conducted with MrBayes version 3.1.2 (Huelsenbeck et al., 2001), using the model of sequence evolution obtained from MrModeltest (GTR+G). Two Markov chains were run simultaneously for 20 million generations and sampled every 200 generations, generating 100,000 trees; 25% of the trees were discarded, and the remaining 75,000 trees were used to construct a majority consensus tree (Fig. 2).

Statistical analyses

Statistical analyses were done with SAS statistical software (SAS, 1999), using generalized linear models with binomial distribution of errors and logit link function.

In the first step, we investigated the relationship between the infection status (dependent, binary variable) and explanatory variables using all individuals in the same model (n = 213 individuals). Explanatory variables were the site (factor), species (binary variable), and first order site–species interaction. In a second step, we performed additional analyses by considering each species separately. For the olive sunbird, we tested the relation between the infection status (dependent, binary variable) and explanatory variables, i.e., the site (factor), sex (binary variable), and the body mass. Finally, we tested the relation between the intensity of infection (parasitemia) and the rate of co-infection with the same explanatory variables described above. For the yellow-whiskered greenbul, we performed the same statistical analyses (without the sex variable).

We performed a statistical test for agreement between microscopy and molecular analysis for both *Plasmodium* and *Haemoproteus* spp. prevalence. We report the Kappa coefficient, confidence interval (CI), and the *P*-value of the test for the null hypothesis that the agreement is purely by chance.

RESULTS

Comparison between microscopy and PCR-based methods

Based on microscopy and PCR methods, we found some discrepancy between the percentage of infected birds (Table III). For the yellow-whiskered greenbul, both methods were concordant for *Plasmodium* spp. (Kappa test agreement = 0.59, CI: 0.39–0.78; Test of H_0 Kappa = 0, $P = 0.0001$) and *Haemoproteus* spp. infection (Kappa test agreement = 0.89, CI: 0.74–1.0; Test of H_0 Kappa = 0, $P = 0.0001$). For the olive sunbird, however, the PCR method underestimated the number of infected birds with *Plasmodium* spp. (Kappa test agreement = 0.28, CI: 0.17–0.42; Test of H_0 Kappa = 0, $P = 0.0001$). This was mainly because PCR did not detect co-infections of haemosporidians, which were numerous in this bird species. With microscopy, compared to PCR methods, we found up to 61% of birds with more than 1 haemosporidean species. The majority of co-infections of *Plasmodium* parasites were detected at the subgeneric level, i.e., species of *Novyella*, *Haemamoeba*, and *Giovannolaia*, which are readily distinguishable in blood films. Thus, to reflect the number of infected individuals and co-infections, we took advantage of the combination of both microscopy and PCR-based methods for the following results.

Parasite lineages diversity and morphospecies

Overall (3 sites, 213 individuals screened), we found 16 distinct mitochondrial lineages of haemosporidian parasites (12 *Plasmodium* spp. and 4 *Haemoproteus* spp.; Fig. 2). The number of different parasite lineages per site varied from 9 in site 1 and 7 in site 2, to 13 in the least-disturbed site 3.

Considering each bird species, we found no significant difference in the diversity of parasite lineages. Namely, we found 6 different *Plasmodium* lineages and 1 *Haemoproteus* lineage (*Haemoproteus vacuolatus* hANLA1, 9.2% of infected birds) in the yellow-whiskered greenbul. Actually, 1 *Plasmodium* lineage largely predominated the parasite community in this host, i.e., *Plasmodium globularis* pANLA1, representing 13.7% of total infections, whereas only 2.7%, 1.8%, 0.9%, 0.9%, and 0.91% of the infections were of the lineages *Plasmodium* PV1L, *Plasmodium* PV3L, *Plasmodium* PV12L, *Plasmodium* PV22L, and *Plasmodium* PV29L.

Similarly, in olive sunbirds, we also found 6 different *Plasmodium* and 3 *Haemoproteus* lineages. *Plasmodium megaglobularis* pCYOL1 was the most prevalent with 14.4% of total infections, whereas in only 3.8%, 1.9%, 1.9%, 1.0%, and 1.0%, the infections were caused by the lineages *Plasmodium lucens* pCYOL2, *Plasmodium* PV13L, *Plasmodium* PV19L, *Plasmodium* PV20L, and *Plasmodium* PV27L, respectively. We also found 21.1% of infected birds with *Haemoproteus cyanomitrae* HV1L, 14.4% with *Haemoproteus cyanomitrae* HV2L, and 2.9% with *Haemoproteus cyanomitrae* HV4L.

Phylogenetic analysis

For the yellow-whiskered greenbul, we found marked host specificity with 1 apparent clade A (Fig. 2) of *Plasmodium* spp. (5 out of 6 lineages grouped together), with the exception of *Plasmodium* PV12L. However, when we used BLAST on this latter sequence, it grouped with shorter, identical sequences (*Plasmodium* PV12; Genbank DQ508387 and *Plasmodium* pGRW9; Genbank DQ060773) found in 19 different avian species, suggesting the strong generalist nature of this lineage.

The *Plasmodium* spp. lineages found in the olive sunbird grouped into 2 different clades: clade B, including *P. megaglobularis* and clade C, including *P. lucens* (Fig. 2). One lineage, *Plasmodium* PV27L, was found in 1 olive sunbird and was grouped with clade A.

Finally, *H. vacuolatus* was found only in the yellow-whiskered greenbul, and *H. cyanomitrae* sp. lineages from olive sunbird grouped together in the clade E.

Prevalence of infection

Prevalence of infection for the 2 parasites species varied among sites in the same avian hosts (Table II). First, we combined all parasite lineages of each genus, and we found a significant interaction between the site and host species for *Plasmodium* spp. ($\chi^2 = 8.81$, $P = 0.012$). Prevalence of *Plasmodium* spp., as well as

numbers of co-infections (Fig. 3) for olive sunbirds, varied among sites, but remained constant for yellow-whiskered greenbuls. In addition, the interaction between the site and host species for *Haemoproteus* spp. (χ^2 = 10.58, P = 0.005) was due to the presence of infected yellow-whiskered greenbuls at only site 3. In addition, we performed tests on separate parasite lineages (when the lineages were more prevalent than 10%). We did not find a site effect on the prevalence of *P. megaglobularis* pCYOL1 or of *H. cyanomitrae* HV1L and HV2L. However, we found a site effect on *H. vacuolatus* hANLA1 prevalence, as we found this lineage only at site 3.

Co-infections and intensity of parasitemia

Co-infections were detected by microscopic observation. Statistical tests were done only for the olive sunbird because co-infections were rare in the yellow-whiskered greenbul (2.75%). First, we found a significant site effect (χ^2 = 21.0, $P < 0.0001$; Fig. 3a) on the detection of single and co-infections, i.e., 0: non-infected, 1: single infection, and 2: co-infection. We also did tests separately for each case of co-infection, i.e., *Plasmodium–Plasmodium* spp. (14.4% of the olive sunbird were co-infected), *Plasmodium–Haemoproteus* spp. (23.1%), and *Haemoproteus–Haemoproteus* spp. (2.9%). We found a significant site effect on *Plasmodium–Plasmodium* spp. co-infections (χ^2 = 10.23, P = 0.006; Fig. 3b), and a marginal effect for *Plasmodium–Haemoproteus* spp. co-infections (c^2 = 5.32, P = 0.07; Fig. 3b).

Intensity of parasitemia in all studied birds varied from just a few parasites in a blood film to 1%, so all infections can be classified as chronic. We found a significant site effect on the chronic parasitemia intensity of *Plasmodium* spp. ($F_{2,101}$ = 3.04, P = 0.05; Fig. 4), but not on the parasitemia intensity of *Haemoproteus* spp. ($F_{2,101}$ = 1.04, P = 0.35) for the olive sunbird. Interestingly, we also found a significant site effect on the parasitemia intensity of *Plasmodium* spp. for the yellow-whiskered greenbul ($F_{2,106}$ = 4.05, P = 0.02), but with the opposite pattern (interaction site–species: $F_{2,210}$ = 5.93, P = 0.0031; Fig. 4). Indeed, for the yellow-whiskered greenbul, the parasitemia intensity was higher at site 1, which is the inverse of that found for the olive sunbird. Sex and body mass did not influence infection prevalence or parasitemia intensity for either of the 2 bird species.

DISCUSSION

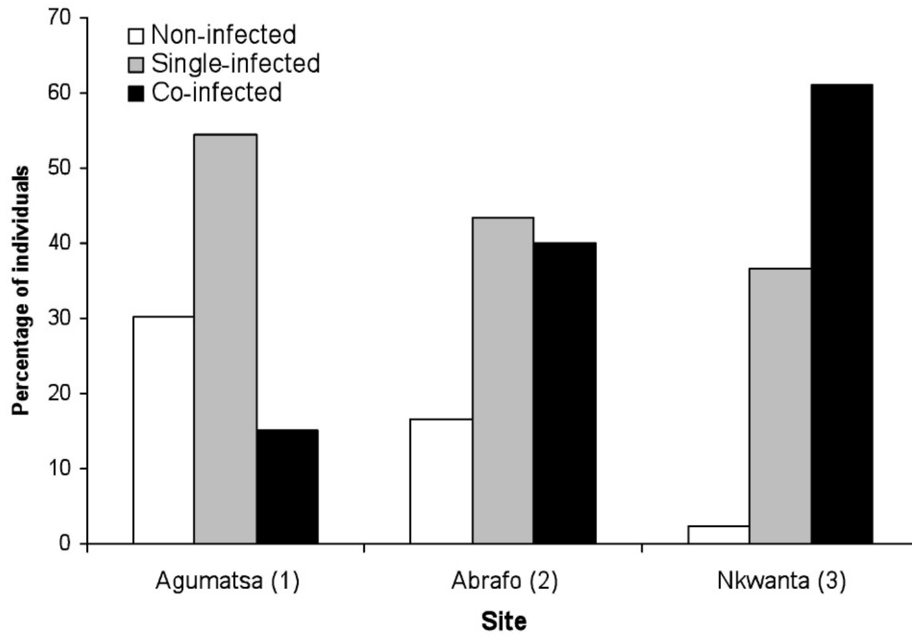
Understanding relationships and interactions in a host–parasite system remains a complex topic in evolutionary ecology. Changes in distribution and prevalence of parasites remain difficult to predict with spatial heterogeneity, as each host–parasite system is susceptible to many uncontrolled variables. In the present study, by taking advantage of both microscopy and PCR-based methods and by controlling for avian species and season, we detected a variation of infection prevalence and intensity of parasitemia at 3 sites that clearly differed in environmental characteristics.

It is worth noting that this study highlighted an important methodological point. It is clear that we still have a need for both PCR and microscopy methods to screen blood samples, as PCR in some cases fails to detect co-infection by different parasites lineages belonging to the same, and even to different, subgenera or genera (Perez-Tris and Bensch, 2005; Valkiūnas et al., 2006; Valkiūnas, Iezhova, Loiseau et al., 2008; Martínez et al., 2009). Co-infection of haemosporidian parasites is common in birds; indeed, we found a high number of co-infections in the olive sunbird (up to 61% at site 3). Thus, it is important to maintain and develop knowledge in traditional parasite taxonomy. We suggest that, currently, both microscopy and PCR methods should be performed in parallel, for all samples, to gain an accurate estimate of parasite prevalence and diversity in studies of ecology and evolutionary biology. To develop precise molecular diagnostics of avian haemosporidians, it is imperative to link morphological characteristics and DNA sequence data in the description and identification of novel parasite morphospecies, (Palinauskas et al., 2007; Valkiūnas, Iezhova, Krazanauskiene et al., 2008; Iezhova et al., 2009; Križanauskiene et al., 2009; Valkiūnas et al., 2009).

Clearly, more sites would be necessary to draw more rigorous conclusions; however, this study conveys interesting patterns. First, in the olive sunbird, we found that the prevalence of *Plasmodium* spp., and the number of co-infections, varied among the sites—sites which exhibited spatial heterogeneity and different climatic variables, with a higher prevalence at site 3. In addition, *Haemoproteus* spp. in the yellow-whiskered greenbul were found only at site 3. Of the 3 sites, site 3 showed the highest percentage of tree cover and the highest NDVImax, as well as the highest levels of precipitation in the wettest or driest quarter, and a higher backscatter. We may conclude that the higher prevalence of infection, and the number of co-infections, are correlated with the different climatic and habitat characteristics described above. These results are in accordance with previous studies in Cameroon (Bonneaud et al., 2009; Chasar et al., 2009), which described a similar pattern of higher parasite prevalence in mature forest as compared to disturbed habitat. These results suggested that several, non-exclusive hypotheses regarding the vector ecology, i.e., breeding sites, the modification of vector competition, or their feeding habits may contribute to vector or parasite variability, or both. Indeed, land changes may influence vector habitat and diversity, distribution, or abundance, but clear patterns cannot be discerned without complete knowledge of each vector's ecology. Although few vector species of *Plasmodium* spp. have been recently investigated in Cameroon (Njabo et al., 2009), vectors and their ecology are, so far, almost unknown for the majority of avian haemosporidian species, particularly in topical ecosystems and especially for *Haemoproteus* spp. Accordingly, there is a crucial need for vector research in Africa. Indeed, in our study, the next step would be to investigate the ecology of biting midges, vectors of *Haemoproteus*, to understand the absence of these parasites at 2 of the 3 sites. A comprehensive investigation on vector abundance and specificity, in interaction with habitat characteristics, is necessary to clarify these findings and to more fully understand the vector's role in host–parasite relationships.

According to our data, intensity of chronic *Plasmodium* spp. parasitemia differed between the 2 species. The chronic parasitemia intensity varied significantly from site 1 to site 3 for the yellow-whiskered greenbul, and we observed an opposite pattern for the olive sunbird. A complex set of factors could influence these results. Intrinsic factors to birds, such as immune characteristics, life history traits, and body condition, are known, for example, to contribute to the variation in parasite prevalence and intensity (Norris and Evans, 2000; Lee et al., 2006; Arriero

a.



b.

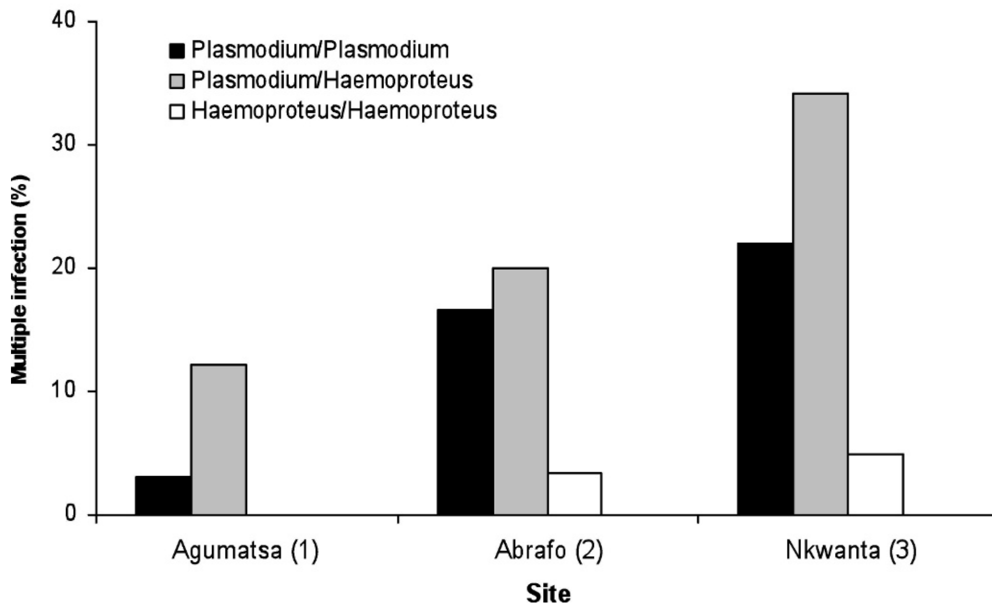


FIGURE 3. (**a**) Percentage of olive sunbird (*Cyanomitrae olivacea*) individuals non-infected (white bar), single-infected (grey bar), and co-infected (black bar), by site. (**b**) Percentage of olive sunbird co-infected individuals, by site, for the 3 cases: 2 or more *Plasmodium* spp. (black bar), 1 or more of each genera (grey bar), and 2 or more *Haemoproteus* spp. (white bar).

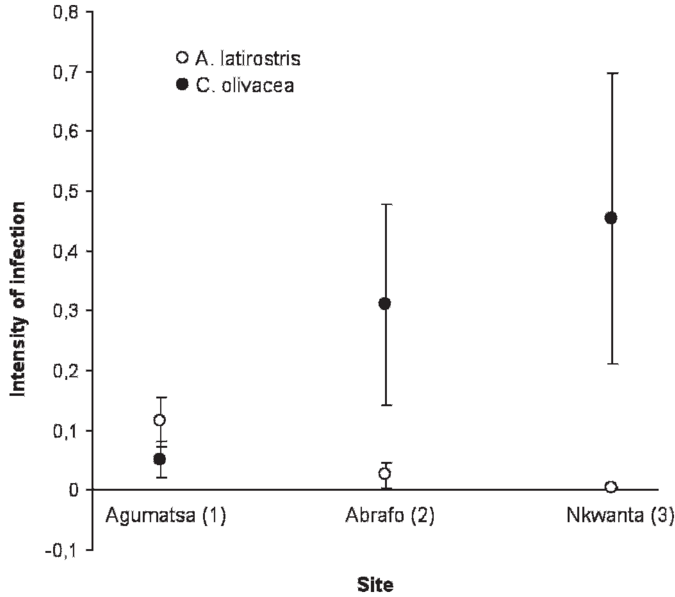


FIGURE 4. Parasitemia intensity ($\log_{10}$) of *Plasmodium* spp. infection (mean ± SE) by site and by bird species.

and Moller, 2008). Although the yellow-whiskered greenbul individuals exhibited nearly the same prevalence of *Plasmodium* spp. at all sites, parasitemia intensity was slightly higher at site 1. In contrast, the olive sunbird showed higher chronic parasitemia intensity at site 3. One possibility is that these 2 species most likely exhibit different strategies for investment in their immune system. Råberg et al. (2007) demonstrated that virulence may give rise to the evolution of an alternative strategy, i.e., tolerance. Further studies should plot parasite burden against different health variables such as haematocrit values, anemia index, the T-lymphocyte-dependent immune response, abundance of ectoparasites, and carotenoid concentration.

We could also suggest that the olive sunbird may have a weaker resistance, i.e., the ability to limit parasite burden, and a lower investment in the immune defence than does the yellow-whiskered greenbul. One hypothesis is that the olive sunbird invests highly in reproductive effort or foraging and, consequently, due to the trade-off between immune system and reproductive effort (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Tomas et al., 2007), may have a higher prevalence of both parasites and the intensity of parasitemia. In this study, it also appears that for olive sunbird individuals, co-infection rates increased parasite virulence, i.e., higher parasitemia was recorded. One explanation suggested by Marzal et al. (2008) was that co-infected individuals maximize current reproduction (larger clutches and more nestlings) when survival prospects are challenged (Bonneaud et al., 2004; Hanssen, 2006; Velando et al., 2006). Unfortunately, there is no information regarding the virulence of these particular parasites, or for reproductive effort, fecundity, or survival of our target host species in Africa. Monitoring during the breeding season and experimental infection investigations are both necessary and would be the next step to understand if such trade-offs occur in regard to habitat changes.

In conclusion, the present study suggests that spatial heterogeneity can impact the prevalence, frequency of co-infections, and chronic parasitemia intensity of haemosporidian parasites. However, patterns of infection markedly differed between host species sharing the same habitat. Future investigations on habitat fragmentation and degradation impacts on host–parasite interactions must not be generalized to all host species. Our findings highlight the importance in clarifying the effects of both biotic and abiotic factors on host–parasite interactions and suggest directions for future population studies on parasites and their vectors in African rainforest birds.

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