Research Article ISSN 2639-9466

Nanotechnology & Applications

Effects of Deforestation on Avian Parasitic Co-infections in Recaptured Birds from an African Tropical Rainforest

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Received: 17 January 2020; **Accepted:** 12 February 2020

Citation: Malange Nanyongo Fedo Elikwo, Anong D. Nota, Tchoumbou M. Adele, et al. Effects of Deforestation on Avian Parasitic Co-infections in Recaptured Birds from an African Tropical Rainforest. Nano Tech Appl. 2020; 2(1): 1-13.

ABSTRACT

The impact of environmental changes due to deforestation that gives rise to the spread of infectious diseases remain insufficiently studied, particularly in parasitic co-infection scenarios. The mark-recapture of birds is of particular interest since we can study human-impacted environments and conduct longitudinal studies of infections. Birds in the South West region of Cameroon were sampled prior to deforestation in 2016 and again in 2017 following deforestation in an area slated for palm oil agriculture. The impact of deforestation on parasitaemia, co-infections trends (of four avian haematozoans and the Superfamily Filarioidea) and the relationships between the prevalence of co-infection of parasites and microclimatic factors (temperature and relative humidity) in all recaptured birds were analyzed using both microscopy and PCR techniques. A total of 1798 birds were caught, 156 of which were recaptures. The three most abundant birds recaptured were Bleda notatus (20.51%), Alethe castanea (18.59%) and Stiphrornis erythrothorax (8.97%). 90.39% of recaptures harbored at least one parasite genus and 81.56% had co-infections. Plasmodium, Trypanosoma and microfilariae parasitaemia, did not change significantly while Haemoproteus and Leucocytozoon parasitaemia varied significantly in particular bird species from first capture to subsequent recapture. Plasmodium exhibited the highest diversity, prevalence and prevalence of co-infection with other avian haematozoans, and differed significantly across both forest types. Random forest analysis revealed that year of sampling, temperature and relative humidity are important predictors of parasitic co-infections. This study recorded fourteen new genetic cytochrome b lineages (10 Plasmodium and 4 Haemoproteus). Our work suggests that of the parasites tested, avian Plasmodium spp. are the best indicators of environmental disturbance because prevalence of infection varied significantly across forest types. Being in the early stages of understanding the complex interactions between avian hematozoa and their hosts in light of rapid environmental change, the study provides baseline information of parasitic co-infection trends in response deforestation.

Keywords

Deforestation, Avian hematozoa, Recaptured birds, Co-infections.

Introduction

Human activities have led to the demise of many bird species, both intentionally [1] and unintentionally [2-4]. The greatest impact on birds has been human activities such as deforestation for agricultural expansion, road construction and urbanization into their natural habitats [5-7]. Birds are an important model for the study of the evolution and ecology of disease and they serve as reservoirs for many types of haemoparasites, which often exist as co-infections in their hosts [8]. Previous studies by Lutz et al. [9] reported 52.7% of co-infections from Malawian birds and 35.9% was reported by Clark et al. [10] in New Caledonian birds. These co-infections may be important for shaping parasite virulence and drug resistance since they provide insights into host parasite co-evolution [11-14] by either increasing [15,16] or decreasing [17,18] parasite virulence. The effects of deforestation on co-infections, which are common in wildlife and predominate in some avian populations have been less studied in tropical rainforests [19,20]. The Democratic Republic of Congo is the country with the highest rate of deforestation, followed by Cameroon of the Congo

Basin countries [21]. Environmental changes alter transmission of endemic pathogens of wildlife and can also lead to the emergence of new pathogens in wild and domesticated animal populations as well as humans [22]. The Talangaye rainforest in the South West region of Cameroon is undergoing rapid deforestation, for the development of palm oil plantations, and is representative of the current deforestation crisis in the country. This deforestation will not only reduce biodiveristy of the bird species it will also have an effect on vectors thereby affecting the outcome of infections. Capture-mark-recapture studies are powerful tools not only for estimating species abundance, survival and population growth rates [23], they can also be used the follow up the progress of infections over time. Our basis for recapturing birds in this longitudinal study was to gain information on the parasitaemia and prevalence of infection and to determine which infections were gained and which were lost in real time following deforestation. Although much is known about avian malaria, its transmission, its genomics, and its drug interactions [8], relatively little is known about how rapid ecological changes affect the transmission of the disease in real time, particularly in co-infection scenarios with other avian blood-borne infections.

Previous studies have investigated the effects of deforestation on multiple avian blood-borne parasites [19,20,24-31] and a few studies have investigated the environmental determinants of the prevalence and variation of co-infections over a large scale [10,32,33] but no study has reported data on the prevalence, variation and co-infection of parasites in response to deforestation. Parasitic co-infections are important since the infection of a host with multiple parasites greatly affects the outcome of each infection when they act synergistically [16,26] or antagonistically [34]. In theory, the most successful parasite competitor would most effectively invade and utilize host resources at the lowest host fitness cost and thereby successfully evade the host immune response [13].

Many avian studies have revealed low prevalence of filarial nematodes, in comparison to the prevalence of other avian haemoparasites [35-38] but none has correlated this to the possibility of co-infection with other parasitic infections. Previous studies by Oakgrove et al. [32] found that the prevalence of *Leucocytozoon* was positively associated with the prevalence of *Trypanosoma* and negatively associated with *Haemoproteus* infections.

Here we study four avian blood-borne parasite genera: *Plasmodium* spp., *Haemoproteus* spp., *Leucocytozoon* spp., *Trypanosoma* spp and the Superfamily Filarioidea in all recaptured birds of the Talangaye rainforest following deforestation in Cameroon. Our longitudinal study investigated how rapid ecological changes affect the transmission of avian blood parasites in real time following deforestation, particularly in co-infection scenarios with other parasitic blood borne infections in recaptured wild birds of the Talangaye rainforest, South West region of Cameroon. We set out to determine: i) the prevalence variation of *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, *Trypanosoma* and filarial nematode parasites in all recaptures; ii) assess the impact of deforestation on parasitemia and co-infections trends; iii) to establish the relationships between prevalence of co-infection of parasites and microclimatic factors; temperature and relative humidity across seasons using random forest models.

Methods

Study Site

This study was carried out in the Talangaye rainforest, Nguti subdivision (5°08'to 5°20'N and 9°22'to 9°24'E), Koupé-Manengouba Division, in the South West region of Cameroon. The Talangaye rainforest has been undergoing large-scale deforestation, for the development of a palm oil plantation. Authorization and access to this area was obtained from Sithe Global-Sustainable Oils Cameroon (SG-SOC), South West region of Cameroon (Figure 1). Talangaye rainforest is located in the midst of the largest remaining contiguous forest block of the West African biodiversity hotspot [39], which covers most of the biogeographic region known as the Gulf of Guinea forests [40].

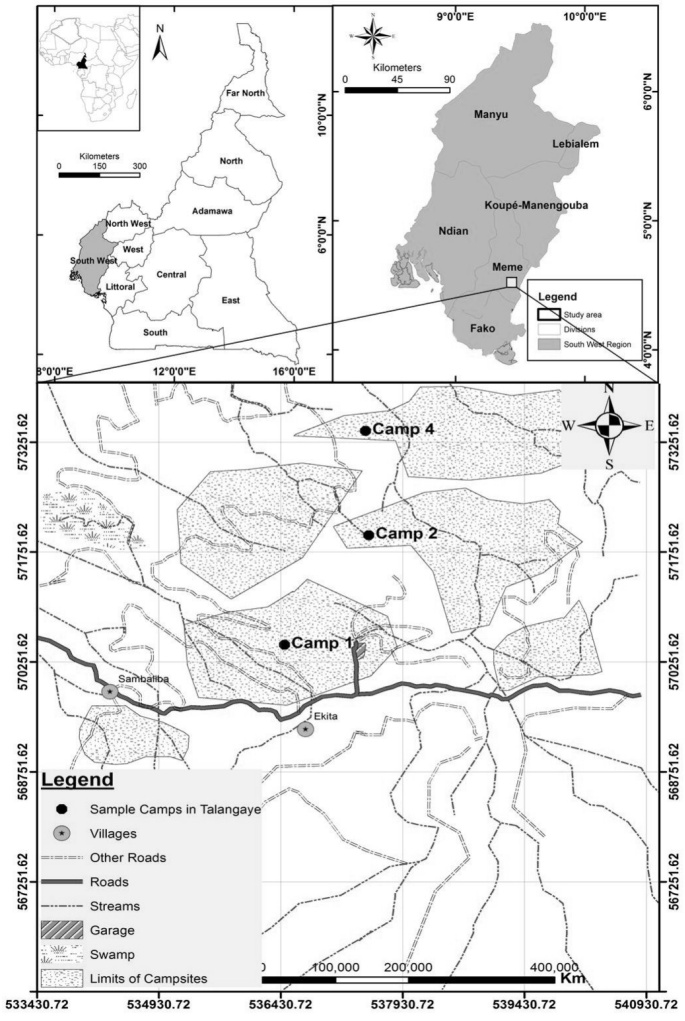

Figure 1: Map showing the sampling areas in the tropical rainforest of Talangaye. All camp sites were sampled twice (in the years 2016 and 2017) using the traditional mist netting technique.

Data was collected during three-week field trips in three camp sites designated camp 2, camp 1 and camp 4 in 2016 in the months of

January (Dry season), April (beginning of rainy season) and July (rainy season) respectively based on the logging plan of SG-SOC. Sampling and identification was repeated in same locations in the year 2017, following deforestation. In the year 2016 all camp sites where pristine forest (intact forest) and in 2017, Camp 1 and 2 had undergone selective logging (fragmented forest) of commercial trees. Therefore Camp 1 and 2 were selected as 'fragmented forest' (defined as forest patch slightly fragmented due to selective logging, it was characterized by the presence of tree stumps, felled trees and road openings due to evidence of human disturbance such as logging for availability of commercial wood and farming) and camp 4 as 'pristine forest' (defined as a forest with mature and tall trees showing little or no evidence of daily human activities). Within each camp, sampling sites differed in bioclimatic predictors (temperature and relative humidity) and habitat characteristics. All sites were between 300m and 400m above sea level (asl).

Field Methods

Bird sampling and blood collection

Three weeks of field work involved sampling of birds by mist netting. Captured birds were weighed, identified, measured, ringed, bled, and released alive. The blood was taken by puncturing the brachial vein. Two thin blood films were prepared per bird on clean glass slides. The smears were then air-dried within 5-15 sec after their preparation with the aid of a battery operated hand fan [8]. The dried slides were then fixed in absolute methanol in the field for at least 1 minute on the same day of their preparation and packed into slide boxes, so that they were well protected from insects and dust. About 50µL of the brachial venipunctured blood of each bird was collected into cryo tubes containing lysis buffer (10mM Tris-HCl pH 8.0, 100mM ethylene-diaminetetraacetic acid, 2% sodium dodecyl sulphate) for subsequent molecular analysis [41]. All smears packed into slide boxes were subsequently transported to the Clinical Diagnostic Laboratory of the University Buea, Cameroon. Sampling of birds was conducted during five days at each site, using on average 16 mist nets (12m long and 2.6m high, 30×30mm mesh with 4 shelves) that were opened at dawn for at least 6 hours and closed during rain. Also, a Geographical positioning system (GPS) was used to record and mark coordinates of the various study sites.

HOBO U23 Pro v2 External Temperature/Relative Humidity Data Loggers were used to record daily temperature and relative humidity of each sampling site. The birds captured were identified using Borrow and Demey [42] and avian taxonomy used conforms to Sibley and Monroe [43]. Captured birds were banded with aluminum numbered rings for ongoing demographic and selection studies and released alive following methods described by Smith et al. [44]. Furthermore, the number of bird species caught and families, as well as the total length of net and number of hours mist nets were opened was noted at each sampling site. All birds captured, novel or recaptures, were recorded but sampling was only conducted once per year. However, note was taken of such recaptured birds when caught the next year of sampling at that site to follow up the parasitemia after 1 year.

Laboratory Methods

Microscopic analysis

The methanol fixed thin blood smears were stained with Giemsa as described by Valkiūnas et al. [8] at room temperature for 90 minutes and microscopy was done according to Valkiūnas et al. [45].

In order to determine the intensity of infections, the number of parasites were counted per 1,000 red blood cells or per 10,000 red blood cells if infections were light (that is < 0.1%), as recommended by Godfrey et al. [46]. Magnifications of 200x and 400x were used to detect microfilariae as described by Sehgal et al. [38].

Polymerase chain reaction (PCR), sequencing analysis

In order to determine the true species composition of the avian blood borne parasites in each naturally infected individual host, a combination of both microscopy and PCR-based methods were used [47-49].

We extracted total genomic DNA following a DNeasy kit protocol (QIAGEN, Valencia, California). The total genomic DNA was used to run four separate PCR reactions in this study, three of which were nested PCR (*Plasmodium*/*Haemoproteus*, *Leucozytozoon* and Trypanosome) [50-53].

For the confirmation of the presence of *Plasmodium*/*Haemoproteus* parasites, a nested PCR approach as described by Waldenström et al. [51] was used to distinguish *Plasmodium* or *Haemoproteus* infections from *Leucocytozoon* infections using genus-specific nested primers, which amplify a fragment of the cytochrome b gene of the parasite. The primers sets were as follows: Nest1 Primers set for *Plasmodium*/*Haemoproteus* was HaemNF/HaemNR2 which amplified gene segments that are similar in both parasite species; while Nest2 Primers set was HaemF /HaemR2 which amplified specific gene segments to *Haemoproteus*/*Plasmodium* spp: [50,52]. For *Leucocytozoon* parasites, Hellgren et al. [52] reaction protocol was employed with Leuco.NFI/Leuco.NR3 as the nested 1 Primer set and Leuco.FL/Leuco.R2L as the nested 2 primers.

Finally, for the *Trypanosoma* PCR reaction a protocol by Valkiūnas et al. [53] was adapted using Tryp763 / Tryp1016 as the nested 1 primers and Tryp99 /Tryp957 as the nested 2 primers to amplify SSU rRNA fragments.

All PCR reactions were run with a set of one positive and one negative control. The positive controls were taken from infected birds, as determined by microscopic examination of blood films, and sterile nuclease free water was used as the negative control, so as to control for false amplification [48]. DNA amplification was done using a BioRad T100™ thermal cycler. All PCR reaction were carried out in a 25µL reaction volume consisting of PuReTaq™ Ready-To-Go™ PCR Beads (Illustra™, GE Healthcare UK), 23 µL master mix (1µL of forward,1µL reverse primer and 21 µL PCR grade water) and 2 µL of template DNA. Furthermore, the PCR products were later subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide and 1µL of loading dye (LGC Biotechnology®) for visualization of the amplified fragment. The

gels were run for 1hr at 150 V and 108 A. To determine the size of the fragment 5µL of 100 bp DNA ladder (AMRESCO®) was used. The gels were visualized and photographed under ultraviolet light using a transilluminator (BIORAD Gel Doc™). Positive or negative infections were seen as the presence or absence of bands of approximately 524 bp for *Plasmodium*/*Haemoproteus*, 480 bp for *Leucozytozoon* and 770 bp for *Trypanosoma*. Following visualization of bright bands, 15µL of all nest 2 products of positive infections were sent for bi directional sequencing on ABI 3730XL DNA Analyzer, by BIONEER, South Korea.

Ethical consideration

The study was approved by the Animal Experimentation Ethics Committee of the University of Buea. Protocols used have been reviewed and accepted by United States Agency for International Development (USAID) through Partnerships for Enhanced Engagement in Research (PEER, project 4-360). A risk assessment was performed before each field trip and appropriate equipment provided for each participant. Required local administrative clearances were obtained to carry out this study in the Talangaye forest of Nguti in South West region of Cameroon.

Data Analyses

The free software R [54] was used to perform all analysis. To avoid bias in detecting prevalence in host species with different abundances, Jovani and Tella [55] suggest using host species with a minimum sample size of 15 individuals.

Analysis of Co-infections

Two co-infection scenarios were considered during analysis:
In the first case: When more than one lineage of a parasite genus (*Trypanosoma*, nematode microfilaria, *Leucocytozoon*, *Plasmodium* or *Haemoproteus*) infects a host simultaneously. In cases of *Plasmodium* and *Haemoproteus* co-infections since the PCR reaction for *Plasmodium* and *Haemoproteus* co-infection does not usually amplify both in the same PCR reaction, the only way to fully differentiate among such co-infections is to clone as suggested by Van Rooyen et al. [56] which was not feasible in our study. Since samples were collected in remote field locations, culturing of the parasites was impractical [41].

In the second case: When multiple lineages from the same genus infect a host simultaneously. By visual inspection of the chromatogram, co-infection is apparent as double-base callings [57]. The identity of the parasites involved was assessed by comparing the double peak patterns with the previously known and highly studied parasite lineages infecting *Eurillas latirostris*, *Cyanomitra olivacea* and *Alethe castanea*.

A Fisher's exact test was performed to determine differences in the frequency of lineage identity changes and infection status (infected vs. uninfected) changes between *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, *Trypanosoma* and nematode microfilaria infections in all recaptured individuals in order to determine which genus was more prevalent before and after deforestation. Generalized Linear Models (GLM) were used to compare prevalence of each parasite genus from one year to another to determine whether an individual's prevalence changed between years of capture.

Linear mixed models (lmer) in the lme4 package [58] were performed on parasitemia data to determine whether there was a relationship between concurrent infection with *Plasmodium* and *Trypanosoma* parasitemia. *Plasmodium* parasitemia was considered the response variable with bird identity and year of capture as random factors and *Trypanosoma* infection as fixed effect. Additional linear mixed models were performed to determine whether there was a relationship between concurrent infection with *Trypanosoma*, *Leucocytozoon*, *Haemoproteus* and nematode microfilariae parasitaemia. *Trypanosoma* parasitaemia was considered as response variable with bird identity and year of capture as random factors and *Leucocytozoon* infection as fixed effect.

Phylogenetic Analysis

Sequences were edited, assembled and aligned by eye using Chromas and SeqMan7.1.0 (DNAStar Inc., Madison, WI, USA). BLAST (Basic Local Alignment Search Tool) searches were used to identify matching sequences down to the genus and species (when possible) of the lineages infecting the host. We compared our sequences to previously sequenced infections in the public databases GenBank (National Center for Biotechnology Information, US National Library of Medicine) and MalAvi [59] using BLAST. We used the closest match to determine the parasite genus for each haplotype. Parasites with sequences differing by at least four nucleotide substitution were considered to represent evolutionarily independent lineages as novel and named following MalAvi naming conventions (first three letters of the genus and species of the first bird host species from which the haplotype was sequenced, followed by a haplotype number for that bird species) and registered in GenBank. The new sequences were submitted to Genbank with the accession numbers (MN104955-MN105013) and to MalAvi database All sequences with double peaks were considered as mixed infections. All evolutionary analyses were conducted in MEGA7 [60]. Searches used the bootstrap search option with 1000 stepwise addition replicates using the TBR branch swapping algorithm. In addition, we performed distance analyses using the Kimura 2-parameter distance model for *Trypanosoma*, Tamura-Nei model for *Plasmodium* and *Haemoproteus*, and taxa were joined using neighbor-joining. Simple consensus trees were constructed to summarize the results.

Results

Species of Birds Recaptured and Parasitic Co-infections

After two years of sampling a total of 1798 birds (1013 in the first year and 785 in the second year) were caught and distinguished as 67 species belonging to 26 families. 156 (8.68%) of the birds were recaptured after the first field visit. Forty-eight birds were recaptured in the dry season in camp 2, twenty-nine in the beginning of the rainy season in camp 1 and seventy-nine in the rainy season in camp 4 (Table 1). Camp 4 was sampled in the rainy season and had the highest number of birds captured in 2016 (386/1013) and recaptures in 2017 (79/156). The recaptures were made up of 23

bird species, belonging to 11 families. Out of the 11 families of birds recaptured, 10 of them harbored parasites. Only one family Picidae (one *Campethera nivosa*) was not infected by any parasite genus even after sampling it a second time a year later in 2017. No recaptures varied from their initial capture sites.

Amongst the 156 recaptures, only 15 (9.62%) individuals remained uninfected after both years of sampling while a prevalence of 90.39 % (141/156) was observed with bird species harboring at least one parasite genus and 81.56% had co-infections within the 2 years of sampling.

Of the recaptures, 72 individual habored single infections and 84 individuals harbored avian parasitic co-infection with parasites from two to four genera present in the infected birds. A co-infection prevalence of 26.09% (30/115) recorded in the fragmented forest area significantly differed ($p = 0.01$) from 73.91% (85/115) observed in the pristine forest. The rainy season had a co-infection prevalence of 51.3%, the dry season of 25.22% and the beginning of the rainy season of 23.48%. A significant difference of co-infection prevalence was observed across seasons ($p < 0.01$) and across forest types ($p = 0.01$). Fragmented forest had lower coinfection rates then pristine and the rainy season is when infections are most evident.

In our study the *Plasmodium* prevalence negatively correlated with that of *Haemoproteus* ($r = -0.42$) ($\chi^2 = 55.92$, $p = 7.57e-14$) and positively with Microfilaria ($r = 0.11$) ($\chi^2 = 6.97$, $p = 0.01$) and *Trypanosoma* ($r = 0.17$) ($\chi^2 = 312$, $p = 2.2e-16$). While *Haemoproteus* prevalence positively correlated with *Leucocytozoon* ($r = 0.14$) ($\chi^2 = 5.92$, $p = 0.02$) prevalence. *Leucocytozoon* prevalence positively correlated with nematode microfilariae ($r = 0.12$) ($\chi^2 = 4.52$, $p = 0.03$) and *Trypanosoma* ($r = 0.21$) ($\chi^2 = 6.97$, $p = 0.01$) prevalences.

Finally, nematode microfilariae prevalence positively correlated with *Trypanosoma* ($r = 0.12$) ($\chi^2 = 6.97$, $p = 0.01$) prevalence, negatively correlation with *Haemoproteus* prevalence ($r = -0.05$) ($\chi^2 = 0.90$, $p = 0.34$) and no correlation with *Plasmodium* prevalence was observed.

Of the 115 co-infections observed, A. castanea had 35 recording 30.43% of co-infections (Table 2). Only individuals of this bird species had co-infections with four parasite genera. Two co-infections with four parasite genera were identified (1 *Plasmodium*/ *Haemoproteus*/ Microfilaria/ *Trypanosoma* co-infection and 3 *Plasmodium*/ *Leucocytozoon*/ Microfilaria/ *Trypanosoma* co-infections). The most prevalent co-infection in both forest types and seasons of this study was *Plasmodium*/*Trypanosoma* with a significantly ($p < 0.01$) high prevalence of 34.78% (40/115) in all recaptured birds. Microfilaria/*Trypanosoma* co-infections were not observed in this study.

Random forest analysis amongst parasite co-infections that positively correlated with each other and the environmental

Family	Species	# of species of birds caught in		
		Camp 2 (# caught)	Camp 1 (# caught)	Camp 4 (# caught)
Alcedinidae (2)	2	0	*Alcedo quadribrachys* (1)	*Ispidina lecontei* (1)
Cisticolidae (3)	1	0	0	*Hylia prasina* (3)
Dicruridae (2)	1	0	0	*Dicrurus atripennis* (2)
Estrildidae (2)	2	0	*Parmoptila woodhousei* (1)	*Spermophaga haematina* (1)
Muscicapidae (14)	1	*Stiphrornis erythrothorax* (4)	*Stiphrornis erythrothorax* (3)	*Stiphrornis erythrothorax* (7)
Nectarinidae (8)	1	0	*Cyanomitra olivacea* (3)	*Cyanomitra olivacea* (5)
Picidae (1)	1	*Campethera nivosa* (1)	0	0
Pycnonotidae (79)	9	*Eurillas latirostris* (1)	*Steidigillas gracilirostris* (2)	*Phyllastrephus icterinus* (4)
		Steidigillas gracilirostris (3)	*Bleda notatus* (4)	*Criniger chloronotus* (3)
		Bleda notatus (15)	*Bleda syndactylus* (3)	*Phyllastrephus xavieri* (2)
		Bleda syndactylus (2)	*Phyllastrephus xavieri* (2)	*Bleda syndactylus* (2)
		Phyllastrephus xavieri (2)		*Bleda notatus* (13)
		Calyptocichla serinus (1)		*Eurillas latirostris* (12)
				Steidigillas gracilirostris (4)
				Eurillas virens (4)
Saxacolinae (35)	2	*Alethe castanea* (14)	*Alethe castanea* (3)	*Alethe castanea* (12)
		Chamaetylas poliocephala (1)	*Chamaetylas poliocephala* (3)	*Chamaetylas poliocephala* (2)
Timaliidae (2)	2	*Illadopsis cleaver* (1)	*Illadopsis rufipenis* (1)	0
Turdidae (8)	1	*Neocossyphus poensis* (3)	*Neocossyphus poensis* (3)	*Neocossyphus poensis* (2)
Total (156)	23	48	29	79

Table 1: Recaptured species of birds classified by family (where numbers in brackets represents total number of birds recaptured per host family). Birds of the family Pycnonotidae (Bulbuls) were the most recaptured in all the seasons and they also recorded the highest number of species caught per family. The three most abundant birds recaptured were *B. notatus* (32/156, 20.51%), *A. castanea* (29/156, 18.59%) and *S. erythrothorax* (14/156, 8.97%) in all sampling sites.

								Co-infections identified										
Species of Birds Recaptured	HL	HLM	HLT	HM	HT	PH	PHL	PHMT	PL	PLM	PLMT	PLT	PM	PMT	PT	LM	LT	Total
Alcedo quadribrachys	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Alethe castanea	0	0	1	0	0	0	1	1	1	1	3	1	3	11	12	0	0	35
Chamaetylas poliocephala	0	0	0	0	0	1	0	0	2	0	0	0	0	0	4	0	0	7
Stelgidillas gracilirostris	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0	4
Eurillas latirostris	0	0	1	0	2	0	0	0	1	0	0	2	0	0	7	0	0	13
Bleda notatus	1	0	1	0	2	0	0	0	0	0	0	0	0	0	3	0	0	7
Bleda syndactylus	0	0	0	0	0	1	0	0	1	0	0	1	2	0	3	0	1	9
Criniger chloronotus	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	2
Cyanomitra olivacea	1	0	0	0	0	0	0	0	0	0	0	5	0	0	5	0	1	12
Dicrurus atripennis	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0	3
Illadopsis rufipenis	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1
Neocossyphus poensis	1	1	2	1	2	0	3	0	0	0	0	0	0	0	0	0	0	10
Parmoptila woodhousei	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
Phyllastrephus icterinus	0	0	0	0	0	0	0	0	0	1	0	2	0	0	2	0	0	5
Phyllastrephus xavieri	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0	3
Stiphrornis erythrothorax	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	2
Total	4	1	5	1	6	2	4	1	10	4	3	14	6	11	40	1	2	115
Percent Total Infected	3.5	0.9	4.4	0.9	5.2	1.7	3.5	0.9	8.7	3.5	2.6	12.2	5.2	9.6	34.8	0.9	1.7	

Table 2: Co-infection in recaptures (Where P=*Plasmodium*, H=*Haemoproteus*, L=*Leucocytozoon*, T=*Trypanosoma*, M= Microfilariae). Sixteen species of birds haboured parasitic co-infections out of the twenty three that were recaptured.

variables in our study revealed: year of sampling, temperature and relative humidity as important predictors of parasitic co-infections (Figure 2). The year of sampling was associated with *Plasmodium*/*Trypanosoma* co-infections explaining 9% of its prevalence (Figure 2a). Relative humidity was the important variable explaining 6% of *Plasmodium*/Microfilaria and 6% of *Leucocytozoon*/Microfilaria prevalence (Figures 2b and 2c). Temperature was the most associated variable to *Haemoproteus*/*Leucocytozoon* co-infection explaining 9% of its prevalence (Figure 2d).

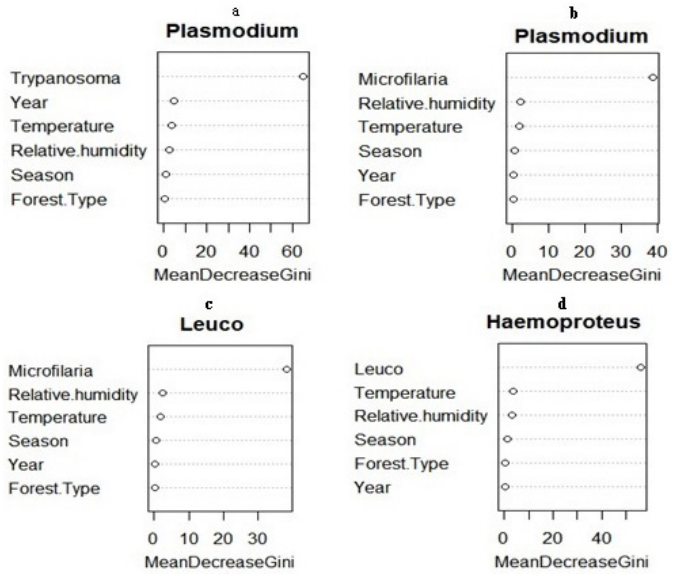

Figure 2: Importance scores for 5 variables related to co-infections prevalences observed in our study determined by random forest models. The year of sampling was the important variable (9%) explaining *Plasmodium*/*Trypanosoma* prevalence (a), relative humidity was the important variable (6%) explaining *Plasmodium*/Microfilaria prevalence (b), relative humidity was the important variable (6%) explaining *Leucocytozoon*/Microfilaria prevalence (c) and temperature was the important variable (9%) explaining *Haemoproteus*/*Leucocytozoon* prevalence (d).

Avian Blood Borne Parasitic Prevalence pre- and post -deforestation

Plasmodium spp. prevalence (64.7%) in Talangaye was found to be higher than that of *Trypanosoma* (20.5%), *Leucocytozoon* spp. (17.3%), *Haemoproteus* spp. (15.4%) and Nematode microfilariae (11.5%) in the first year of capture in 2016. While in the next year following deforestation, the recaptured birds had a *Plasmodium* spp. prevalence of 57.7%, which was higher than that of *Trypanosoma* (37.8%), *Haemoproteus* spp. (16.7%), *Leucocytozoon* spp. (15.4%) and Nematode microfilariae (7.7%) (Table 3). Also see supplementary table material for detailed parasite infection status for individual birds in both years of sampling.

In the pristine forest the prevalence of *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and *Trypanosoma* were highly significant in the species of birds recaptured (χ^2 = 63.58, $p < 0.01$) whereas that of nematode microfilariae was not significant (χ^2 = 37.33, p = 0.06). while in the fragmented forest only *Haemoproteus* (χ^2 = 3.08, $p<0.02$) and *Leucocytozoon* (χ^2 = 26.47, p = 0.04) infections differed significantly among species of birds recaptured. Only the *Plasmodium* parasite genus differed significantly in all recaptures across both forest types following deforestation (χ^2 = 6.06, p = 0.01) and it recorded the highest prevalence in both forest types (65.11% in pristine and 49.35% in fragmented forest) (Figure 3).

The prevalence of the other four parasites in recaptures did not show any significant changes across forest types (p = 0.09).

After deforestation we noticed that infection with *Haemoproteus*, Nematode microfilariae and *Trypanosoma* prevalences increased following recapture, while *Leucocytozoon* and *Plasmodium*, prevalences dropped (Figure 3).

Scientific names	# tested	# infected	Infection status in 2016	Infection status in 2017
			P H L T M	P H L T M
Alcedo leucogaster	1	1	0 1 1 0 0	0 1 0 0 0
Alethe castanea	29	27	23 2 4 13 11	24 2 4 17 8
Chamaetylas poliocephala	6	6	6 1 1 2 0	4 1 1 4 0
Stelgidillas gracilirostris	9	9	9 0 0 0 1	6 0 2 3 1
Eurillas latirostris	13	12	11 1 3 3 0	8 4 1 9 0
Eurillas virens	4	3	2 0 0 0 0	3 0 0 0 0
Bleda notatus	32	28	15 9 0 1 1	16 9 2 6 0
Bleda syndactylus	7	7	6 1 2 2 1	5 0 1 5 1
Calyptocichla serinus	1	1	0 0 1 0 1	0 1 0 0 0
Campethera nivosa	1	0	0 0 0 0 0	0 0 0 0 0
Ispidina lecontei	1	1	1 0 0 1 0	1 0 0 0 0
Criniger chloronotus	3	3	3 0 0 0 0	3 0 1 1 1
Cyanomitra olivacea	8	8	6 1 4 4 0	5 0 3 7 0
Dicrurus atripennis	2	2	2 0 2 0 0	2 0 1 1 0
Hylia prasina	3	3	0 1 0 0 0	2 0 0 0 0
Illadopsis cleaveri	1	1	0 0 1 0 0	0 0 0 0 0
Illadopsis rufipensis	1	1	1 0 0 0 0	1 0 1 0 0
Neocossyphus poensis	8	8	2 7 4 2 1	1 7 4 3 1
Parmoptila woodhousei	1	1	0 0 0 0 0	1 0 0 1 0
Phyllastrephus icterinus	4	4	4 0 2 2 1	2 1 1 2 0
Phyllastrephus Xavieri	6	6	3 0 1 1 0	5 0 2 0 0
Spermophaga haematina	1	0	0 0 0 0 0	0 0 0 0 0
Stiphrornis erythrothorax	14	9	7 0 1 1 2	1 0 0 0 0
Total birds captured	156	141	101 24 27 32 18	90 26 24 59 12
Percent Total Infected	90.4		64.7 15.4 17.3 20.5 11.5	57.7 16.7 15.4 37.8 7.7

Table 3: Parasitic Co-infection prevalence following capture and recapture of bird species (Where P=*Plasmodium*, H=*Haemoproteus*, L=*Leucocytozoon*, T=*Trypanosoma*, M= Microfilariae).

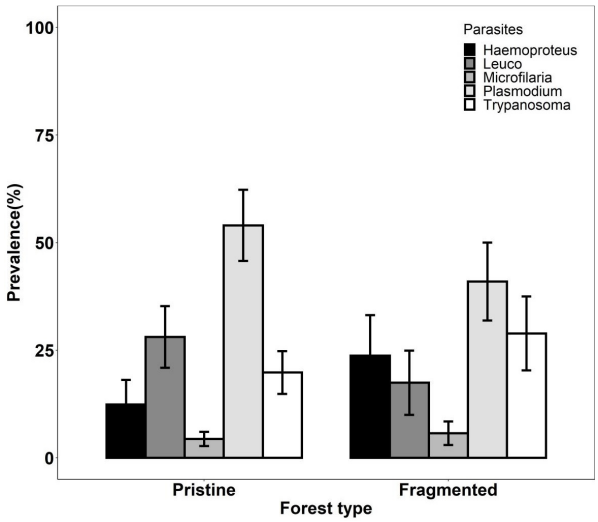

Figure 3: Prevalence of parasites in recaptured birds across forest types.

For *Plasmodium* (t = 0.04, p = 0.97), *Trypanosoma* (t = -0.51, p = 0.61) and microfilariae (t = 0.03, p = 0.97), parasitaemia did not change significantly from first capture to the subsequent recapture. However, *Haemoproteus* and *Leucocytozoon* parasitaemia varied significantly in particular bird species from the first year of capture (2016) to the other (2017) following recapture.

For *Haemoproteus* significant differences were observed in the following bird species: *A. castanea* (t = -3.98, p < 0.01), *C. olivacea* (t = -2.92, p < 0.01), *B. syndactylus* (t = -2.78, p = 0.01), *E. latirostris* (t = -2.54, p = 0.01), *P. icterinus* (t = -2.19 p = 0.03), *C. poliocehala* (t = -2.31, p = 0.02) and *B. notatus* (t = -2.19, p = 0.03).

B. notatus (t = -2.46, p = 0.02) and *S. erythrothorax* (t = -2.02, p = 0.04) were the only two bird species that recorded significant *Leucocytozoon* parasitaemia from one capture to a subsequent capture.

Parasite lineage diversity and phylogenetic relationships

Sequencing results yielded 84 *Plasmodium* lineages (from a total of n = 191 infected with this genus), 19 *Haemoproteus* lineages (n = 58), 2 *Leucocytozoon* lineages (n = 51), 36 *Trypanosoma* lineages (n = 91). No Nematode microfilaria lineages (n=30) were identified due to poor sequence quality. Blast searches in Genbank revealed 18 sequences (submitted with accession numbers MN104955-MN104977) of probably new lineages of *Plasmodium* (10) ad *Haemoproteus* (8) parasites but upon submission to MalAvi database for confirmation 4 were already recorded lineages; 2 PHICT03 and 2 COLL2 (MN104970, MN104971, MN104972 and MN104973). Finally, the fourteen newly discovered lineages (10 *Plasmodium* and 4 *Haemoproteus*) were named following MalAvi naming conventions (Table 4). For *Trypanosoma* 36 lineages with Genbank accession numbers MN104978 -MN105013 were recorded but they were not resubmitted to MalAvi database for confirmation since MalAvi was created only for haemosporidian parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) based on mitochondrial cytochrome b lineages. The phylogenetic

relationships of *Plasmodium*/*Haemoproteus* and *Trypanosoma* trees revealed groups of diverse avian blood borne lineages (Figures 4a and b). Lineages identified in this study infected a variety of host species making them generalist parasites (n = 51) and the most prevalent lineage was P-ALDI1 *Plasmodium* (Novyella) parahexamerium with 19.1% (16/84). This P-ALDI1 lineage had a very high prevalence of 81.3% (13/16) in A. castanea and was also found in three additional hosts namely; *E. latirostris*, *B. notatus* and *D. atripennis*.

SN of lineages	Lineage Name	GENBANK #	Host species
1	CHAPOL01	MN104975	*Chamaetylas poliocephala*
2	CHAPOL02	MN104976	*Chamaetylas poliocephala*
3	EURLAT01	MN104974	*Eurillas latirostris*
4	ALCQUA02	MN104977	*Alcedo quadribrachys*
5	ALECAS01	MN104966	*Alethe castanea*
6	PHYXAV01	MN104964	*Phyllastrephus xavieri*
	PHYXAV01	MN104965	*Bleda notatus*
	PHYXAV01	MN104967	*Calyptocichla serinus*
	PHYXAV01	MN104969	*Bleda notatus*
7	STEGRA01	MN104957	*Steidigillas gracilirostris*
	STEGRA01	MN104968	*Steidigillas gracilirostris*
8	ILLRUF02	MN104963	*Illadopsis rufipenis*
9	PARWOO01	MN104962	*Parmoptila woodhousei*
10	CYAOLI19	MN104961	*Cyanomitra olivacea*
11	STEGRA02	MN104956	*Steidigillas gracilirostris*
12	STEGRA03	MN104955	*Steidigillas gracilirostris*
	STEGRA03	MN104959	*Steidigillas gracilirostris*
13	BLESY02	MN104960	*Bleda syndactylus*
14	CRICHL01	MN104958	*Criniger chloronotus*

Table 4: Newly recorded lineages of *Haemoproteus* and *Plasmodium* from recaptured birds.

Lineages 1-4 are of the *Haemoproteus* genus while 5-14 are *Plasmodium* lineages. The lineage PHYXAV01 was identified in three different host species (*P. xavieri, B. notatus* and *C. serinus*) belonging to the family Pycnonotidae.

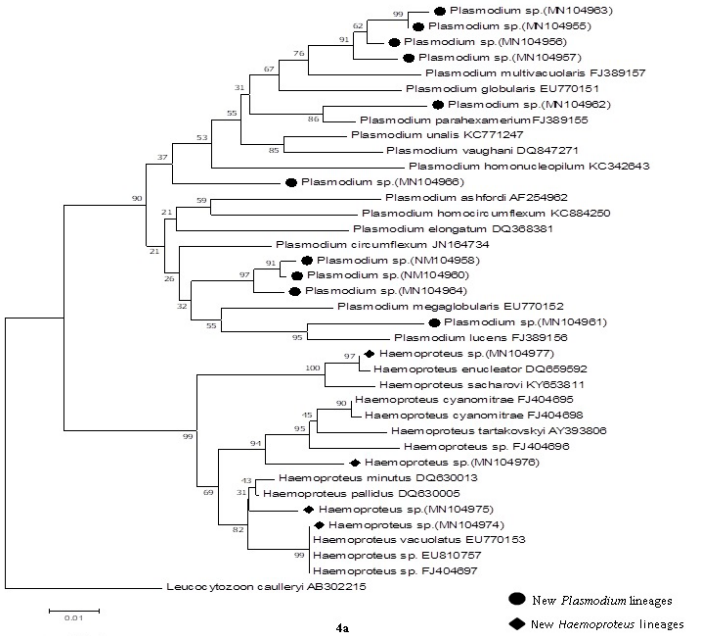

Figure 4a: Phylogenetic relationships among truly distinct *Plasmodium*/*Haemoproteus* cyt b lineages of recaptured wild birds from Talangaye rainforest.

GenBank accession numbers of all newly recorded sequences are indicated in brackets for *Haemoproteus* (4) and *Plasmodium* (10). *Leucocytozoon caulleryi* was used as an outgroup.

Figure 4b: Phylogenetic tree of truely distinct *Trypanosoma* SSU rRNA lineages. Numbers along branches correspond to node support from posterior pobabilities. *Leucocytozoon caulleryi* was used as an outgroup. The host species for the *Trypanosoma* spp. lineages are represented by various shapes in alphabetic order.

Discussion

This study offers the first insight into how rapid ecological changes affect the transmission of avian malaria in real time in recaptured birds. Recaptured birds are important, because they give information on how infections can change in individual birds over time both in their natural and altered environments. The study develops baseline model in predicting how habitat changes may affect parasite distributions and co-infection interactions particularly in co-infection scenarios following deforestation. Our study examines parasitic blood borne infections at the individual level in free-living recaptured birds during three seasons and two forest types following deforestation. Traditional microscopy and PCR detection techniques revealed that co-infections of malaria parasite (haemosporidians) and other parasitic blood-borne infections are common in the rainforest birds of Talangaye, South West region of Cameroon. Parasite prevalence was high 141/156 (90.4%) with only sixteen individuals remaining parasite free during both years of capture. This is consistent with the high detection of infection with multiple parasites in natural populations of other African bird species reported by Valkiūnas et al. [61]. This could be evidence for a more synergistic/benign type of interaction as summarized in Palinauskas et al. [26], where a parasite induces prolonged infection or better establishment of another parasite.

We recorded a co-infection prevalence of 81.56% in recaptured birds. This prevalence is relatively low compared to the results of Van Rooyen, et al. [56] where avian haemosporidian persistence and co-infection in great tits recorded parasite prevalence of 98%. The low parasite prevalence of our study can be explained by the fact that even though 11 families of birds were recaptured, mostly 1-8 individuals were recaptured per family representing 72.7% (8/11) of the data set. Also, this tropical rainforest ecosystem differs considerably from the temperate ecosystem studied by Van Rooyen et al. [56]. Furthermore, this prevalence of 81.56% is relatively high compared to a study by Sehgal et al. [38] in some West African countries that reported an overall prevalence of 28.6% using only microscopy in parasite detection. It is certain that we still have a need for both PCR and microscopy methods to screen blood samples, even though PCR in some cases fails to detect co-infection by different parasites lineages belonging to the same, and even to different, subgenera or genera [47,57,61].

For *Plasmodium* ($t = 0.04$, $p = 0.97$), *Trypanosoma* ($t = -0.51$, $p = 0.61$) and microfilariae ($t = 0.03$, $p = 0.97$), parasitaemia did not change significantly from first capture to the subsequent recapture. However, *Haemoproteus* and *Leucocytozoon* parasitaemia varied significantly in particular bird species from the first year of capture (2016) to the next (2017) following recapture. *Haemoproteus* reduced significantly in the following bird species: *A. castanea* ($t = -3.98$, $p < 0.01$), *C. olivacea* ($t = -2.92$, $p < 0.01$), *B. syndactylus* ($t = -2.78$, $p = 0.01$), *E. latirostris* ($t = -2.54$, $p = 0.01$), *P. icterinus* ($t = -2.19$ $p = 0.03$), *C. poliocephala* ($t = -2.31$, $p = 0.02$) and *B. notatus* ($t = -2.19$, $p = 0.03$). While *Leucocytozoon* parasitaemia significantly increased in *B. notatus* ($t = -2.46$, $p = 0.02$) and *S. erythrothorax* ($t = -2.02$, $p = 0.04$) from one capture to subsequent capture. The reasons for this variation are not known but may be due to the fact that birds vary widely in their susceptibility to infection and their immune systems would be more capable of combatting infections over time. Previous findings have shown that the age of birds [62,63] and any previous or concurrent infections [26] can greatly affect their susceptibility to infection and hence the parasitaemia of infection. Further work is required to explore the influence of these factors on susceptibility to malaria infections. Parasitaemia, however, is correlated with several host factors, including host immunity and metabolic profiles which may affect the likelihood of mosquito infection [64].

In our study, *Plasmodium* exhibited the highest diversity (cyt b lineage richness), prevalence and prevalence of co-infection with other avian haematozoans across seasons and across forest types. This might be due to the tropical climate in the Talangaye forest that favors the vectors of *Plasmodium* spp.(mosquitoes) the most when compared to the other parasites. Co-infections involving *Plasmodium* and *Trypanosoma* parasites were the most prevalent co-infections as opposed to findings by Svobodová et al. [65], where co-infections involving *Leucocytozoon* and *Trypanosoma* parasites were more frequent. We detected *Plasmodium*/*Haemoproteus* co-infections, which could only be achieved by the combination of both microscopy and molecular diagnostic techniques [47].

We found a positive association between haemosporidian (only *Plasmodium* and *Leucocytozoon*) and trypanosome infections, meaning that individuals infected by one parasite are more likely to carry a second protozoan infection. Previous studies by Soares et al. [66] and Oakgrove et al. [32] observed similar relationships between haemosporidian and trypanosome parasites but *Trypanosoma* infections were positively associated with *Leucocytozoon* and *Haemoproteus* infections, and not with *Plasmodium* infections. This might be associated to vector co-transmission. The primary vectors of avian malaria parasites (*Plasmodium* spp.) are Culicidae mosquitoes belonging to eight genera [8]. In addition, several ornithophilic blood-feeding mosquito species within multiple other mosquito genera (Aedes, Anopheles, Mansonia, Aedeomyia) and wild mosquitoes of the genus Coquillettidia have been implicated in the transmission of different species of avian *Plasmodium* Bonneaud et al. [20]; Njabo et al. [67]. We suspect that Aedes and Culex mosquitoes are responsible for transmitting *Plasmodium* here in the rainforest. Schmids et al. [68] recently identified Uranotaemia caeruleocephala as a competent vector of avian malaria in Madagascar. Parasites of the genus *Trypanosoma* are transmitted by a variety of vectors, including simuliids, ceratopogonids and culicids, also known to transmit other avian blood parasites [69]. Furthermore, trypanosomes might be spread by the birds eating the insects, which is very different from *Plasmodium* [41].

A weak association of environmental variables (temperature and relative humidity) and the *Leucocytozoon*/ *Trypanosoma* co-infection was observed in our study. This is opposite to very strong associations revealed by Oakgrove et al. [32] in Alaska. In Cameroon the temperatures are much more stable and consistent than in Alaska. In considering bird families with more than 15

individuals [55], *A. castanea* recorded the highest *Plasmodium*, *Trypanosoma* and Nematode microfilariae prevalence. We found a prevalence of 37.9% (11/29) in *A. castanea*, and no microfilariae in the congeneric *C. poliocephala* (0/6). These results are similar to those by Sehgal et al. [38] where no microfilariae were found in the *C. poliocephala* but with higher prevalence of 62% in A. castanea. The most prevalent lineage (P-ALDI1) *Plasmodium* (Novyella) parahexamerium had a high prevalence of 81.3% (13/16) in the *A. castanea* when compared to a previous study by Valkiūnas et al. [48] of 69.2% (18/26). Additional new hosts for this lineage for the first time was discovered in our study (*E. latirostris, B. notatus* and *D. atripennis*) as opposed to the previous study by Valkiūnas et al. [48] wherein A. castanea was the only host.

We submitted 36 *Trypanosoma* sequences to Genbank but upon construction of phylogenetic trees only 16 lineages were truly distinct from each other (Figure 4b). Unlike Avian haemosporidians there is a urgent need for a unified database of avian trypanosmes lineages.

The fact that all the five parasites in our study were identified in all three seasons though with varying prevalences supports the knowledge that in tropical climates, avian malaria occurs year-round [8], whereas studies in temperate regions report consistent seasonal variation: a peak in prevalence during spring or the breeding season, followed by a decline during winter [70-73].

In the rainy season the highest number of birds were recaptured and highest parasitic cyt b lineage diversity was recorded. *Plasmodium* and *Trypanosoma* prevalence were highest in the rainy reason, *Haemoproteus* and *Leucozytozoon* were highest in the beginning of the rainy season and nematode microfilariae was highest in the dry season. The rainy season increases the availability and sources of breeding for mosquito communities.

Trypanosomes remain a neglected group of avian blood parasites [33,53]. Light *Trypanosoma* parasite intensity is a big obstacle in field studies of avian trypanosomes [33,41,74,75]. Similarly, high intensities of T. everetti trypomastigotes in peripheral blood was observed in recaptures as in Valkiūnas et al. [30] in tropical African passerines.

Sampling by Bennett et al. [76,77] over a period of 6 years found no significant differences in the overall prevalence of hematozoan infections in birds collected during wet and dry seasons or in different years in Uganda. These results are different from those obtained in this study wherein; *Plasmodium*, *Haemoproteus*, *Leucocytozoon* and Nematode microfilaria parasites did not differ significantly vary across sampling over 2 years, only *Trypanosoma* ($\chi^2 = 16.90$, p = 3.95e-05) parasites differed. This is highly suggestive that; (i) infection with this genus is opportunistic and only establishes when other parasites have invaded a host immunes system, (ii) vectors responsible for *Trypanosoma* transmission fluctuate in abundance according to annual climatic variation (e.g. temperature and relative humidity), which changes the microclimates they require for breeding. Greater transmission rates may thus occur in years when conditions are more favorable for vectors.

Only the *Plasmodium* parasite genus differed significantly in all recaptures across both forest types following deforestation ($\chi^2 = 6.06$, p = 0.02) with pristine forests displaying significantly higher prevalence of infection with avian *Plasmodium* spp. than fragmented forest. These results support the hypothesis that forest density and structure may influence interactions between species and play a role in the transmission and/or maintenance of infections [20]. We further support previous findings by Valkiūnas et al. [30], which suggested that spatial heterogeneity related to deforestation does not affect the prevalence of avian *Trypanosoma* infections. Deforestation does not also affect the prevalence of avian filarial nematode infections. Previous findings by Valkiūnas et al. [30] revealed that the broad specificity and involvement of many species of avian hosts and numerous species in avian trypanosomiasis is probably responsible for forest type having no effect on bird–T. everetti interactions and therefore avian trypanosomes cannot be recommended as indicators of environmental disturbance related to deforestation because transmission shows the same patterns both in pristine and markedly fragmented agroforests in tropical Africa.

Conclusion

Being in the early stages of understanding the complex interactions between avian hematozoa and their hosts in light of rapid environmental change [33], our study provides baseline information of co-infection trends in this part of Cameroon that is undergoing deforestation. Most studies only consider *Plasmodium* and *Haemoproteus* parasites when investigating avian haemosporidian infections and as a result *Leucocytozoon* is underrepresented in the literature. This study has focused not only on *Plasmodium*, *Haemoproteus* and *Leucocytozoon* infection across time at the individual scale, but also considered multiple infections of these parasites and other parasitic blood borne infections (*Trypanosoma* and Nematode Microfilariae) and what that might imply for Haemosporida coevolution with their hosts. Individuals infected by one parasite may be predisposed to infection with a second parasite since parasitic co-infections are common and predominate in avian populations in Talangaye forest of Cameroon. There is an urgent need for a unified database of avian trypanosmes lineages. In conclusion our work suggests that only avian *Plasmodium* can be recommended as indicators of environmental disturbance related to deforestation because prevalence varied significantly across forest types in this tropical rainforest in Cameroon.

Acknowledgments

This work was funded by the United States Agency for International Development-Partnerships for Enhanced Engagement in Research (USAID-PEER) Project 4-360 grant awarded to Dr. Anong Damain Nota. This work also received support from the Conservation Action Research Network Grant (CARN) and Ideawild awarded to Malange N. F. Elikwo. Ravinder N.M. Sehgal's and Anthony J. Cornel's travels to Cameroon were supported by the National

Geographic Foundation (CRE-983616). We also thank the General Manager of the palm oil plantation Company SGSOC, Dr Blessed Okole for granting us permission to collect samples from the plantation sites, Mr Alfred Akumsi for field support and also the forest guides from Manyemen. We are immensely grateful to Mr. Francis Forzi (Ornithologist) for his professional help during field trips as well as all the students of the USAID-PEER project 4-360 who spent so much time in the field collecting data for this project. The students remain indebted to the immense contribution of the international collaborators (Prof. Anthony John Cornel and Dr. Kevin Njabo) who greatly impacted on the nature and outcome of this project. We also thank Mr Mokake Fidelis for drawing the map of sampling sites and Dr. Leke Walters for his immense help on the phylogenetic analysis part of this work.

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