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

Malange Nanyongo Fedo Elikwo¹, Anong D.Nota¹, Tchoumbou M. Adele², Jerome Fru-Cho¹, Tabe T. R. Claire¹, Tibab Brice¹ and Ravinder N.M. Sehgal³

¹Department of Microbiology and Parasitology, University of Buea, Cameroon.
²Department of Animal Biology, University of Dschang, Cameroon.
³Department of Biology, San Francisco State University, San Francisco, CA, USA.

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.
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 biodiversity 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 to 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 sub-division (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.](image-url)
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, Leucocytozoon 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 (Illumia™, 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 1 hr 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 *Leucocytozoon* and 770 bp for *Trypanosoma*. Following visualization of bright bands, 15μL of all nest 2 products of positive infections were sent for bidirectional 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 harbored 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 area significantly differed (p = 0.01) from pristine and the rainy season is (p < 0.01) and across forest types (p = 0.01) prevalence with a significantly (p < 0.01) high prevalence of 34.78% (40/115) in all recaptured birds. Microfilaria/parasites from two to four genera present in the infected birds.

In our study the *Plasmodium* prevalence negatively correlated with that of *Haemoproteus* (r = -0.42) (ɤ² = 55.92, p = 7.57e-14). Finally, nematode microfilariae prevalence positively correlated with *Trypanosoma* (r = 0.12) (ɤ² = 6.97, p = 0.01) prevalence, negatively correlation with *Haemoproteus* prevalence (r = -0.05) (ɤ² = 0.90, p = 0.34) and no correlation with *Leucocytozoon* 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) |
| Dicuridae (2)         | 1                | 0                  | 0                  | Dicrocites aprigenus (2) |
| Estrildidae (2)       | 2                | 0                  | Parmoptila woodhousei (1) | Spermophaga haematina (1) |
| Muscicapidae (14)     | 1                | 0                  | Stiphronis erythrothorax (4) | Stiphronis erythrothorax (3) |
| Nectarinidae (8)      | 1                | 0                  | Cyanomitra olivacea (3) | Cyanomitra olivacea (5) |
| Picidae (1)           | 1                | 0                  | 0                  |
| Pycnonotidae (79)     | 9                | Campethera nivosa (1) | 0                  |
| Saxicolinae (35)      | 2                | Eurillas latirostris (1) | Steidigillas gracilirostris (2) | Phyllostrepus icterusinus (4) |
| Timaliidae (2)        | 2                | Steidigillas gracilirostris (3) | Bleda notatus (4) | Cripiger chloronatus (3) |
|                       |                  | Bleda notatus (15) | Bleda symbatylus (3) | Phyllostrepus xavieri (2) |
|                       |                  | Bleda syndactylus (2) | Phyllostrepus xavieri (2) | Bleda symbatylus (2) |
|                       |                  | Phyllostrepus xavieri (2) | Bleda notatus (13) |
|                       |                  | Calyptocichla serinae (1) | Eurillas latirostris (12) | Steidigillas gracilirostris (4) |
|                       |                  | 0                  | 0                  |
| Saxicolinae (35)      | 2                | 0                  | 0                  |
| Timaliidae (2)        | 2                | Ildadopsis cleaver (1) | Ildadopsis rufipes (1) |
|                       |                  | 0                  |
| Turdidae (8)          | 1                | Neocossyphus poenisi (3) | Neocossyphus poenisi (3) |
| Total (156)           | 23               | 48                 | 29                 |

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 (Bulbul) 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.
Importance scores for 5 variables related to co-infections

Table 2: Co-infection in recaptures (Where P=Plasmodium, H=Haemoproteus, L=Leucocytozoon, T=Trypanosoma, M= Microfilariae). Sixteen species of birds harboured 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).

In the pristine forest the prevalence of Plasmodium spp. was lower (46.7%) in Talangaye was found to be higher than that of Trypanosoma spp. (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 spp. (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.

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 spp. (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 spp. (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 (x² = 63.58, p < 0.01) whereas that of nematode microfilariae was not significant (x² = 37.33, p = 0.06). While in the fragmented forest only Haemoproteus (x² = 3.08, p<0.02) and Leucocytozoon (x² = 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 (x² = 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 |
|----------------------------|----------|------------|--------------------------|--------------------------|
| Alcedo leucogaster         | 1        | 1          | 0 1 0 0                  | 0 1 0 0                  |
| Alceda castanea            | 29       | 27         | 23 2 4 13 11             | 24 2 4 17 8             |
| Chamaeytys poliocephala    | 6        | 6          | 6 1 1 2 0                | 4 1 1 4 0               |
| Stegalella graciilrostis    | 9        | 9          | 9 0 0 0 1                | 6 0 2 3 1               |
| Euarilas lairostris        | 13       | 12         | 11 1 3 3 0               | 8 4 1 9 0               |
| Euarilas virites           | 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 syndactys            | 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               |
| Campephera nivosiss        | 1        | 0          | 0 0 0 0 0                | 0 0 0 0 0               |
| Ispidina leoniti           | 1        | 1          | 1 0 0 1 0                | 1 0 0 0 0               |
| Cringier chloronotus       | 3        | 3          | 3 0 0 0 0                | 3 0 1 1 1               |
| Cyamomirra olivacea        | 8        | 8          | 6 1 4 0 0                | 5 0 3 7 0               |
| Dicrurus atipennis         | 2        | 2          | 2 0 2 0 0                | 2 0 1 1 0               |
| Hylla prasina              | 3        | 3          | 0 1 0 0 0                | 2 0 0 0 0               |
| Ildadunis cleaveri         | 1        | 1          | 0 0 1 0 0                | 0 0 0 0 0               |
| Ildadunis rufipenis        | 1        | 1          | 1 0 0 0 0                | 1 0 1 0 0               |
| Neochorphyys poenisii      | 8        | 8          | 2 7 4 2 1                | 1 7 4 3 1               |
| Paratopita woodhousei      | 1        | 1          | 0 0 0 0 0                | 1 0 0 1 0               |
| Phyloxophus icterus        | 4        | 4          | 4 0 2 2 1                | 2 1 1 2 0               |
| Phyloxophus Xavier         | 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               |
| Stiphenas erythorhoxas     | 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).

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. icterus (t = -2.19 p = 0.03), C. poliocephala (t = -2.31, p = 0.02) and B. notatus (t = -2.19, p = 0.03).

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. icterus (t = -2.19 p = 0.03), C. poliocephala (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 microfilariae 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) and 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.

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

| SN of lineages | Lineage Name | GENBANK # | Host species |
|---------------|--------------|-----------|-------------|
| 1             | CHAPOL01     | MN104975  | Chamaetula poliocephala |
| 2             | CHAPOL02     | MN104976  | Chamaetula poliocephala |
| 3             | EURLAT01     | MN104974  | Eurilla latirostris |
| 4             | ALCQUA02     | MN104977  | Alcedo quadribrachys |
| 5             | ALECAS01     | MN104966  | Alethe castanea |
| 6             | PHYXAV01     | MN104964  | Phyllostomus xavieri |
|               | PHYXAV01     | MN104965  | Bleda notatus |
|               | PHYXAV01     | MN104966  | Calyptocichla serinus |
| 7             | STEGRA01     | MN104957  | Steidigillas gracilirostris |
|               | STEGRA01     | MN104956  | Steidigillas gracilirostris |
|               | STEGRA03     | MN104955  | Steidigillas gracilirostris |
|               | STEGRA03     | MN104959  | Steidigillas gracilirostris |
| 8             | BLESY02      | MN104960  | Bleda syndactylus |
| 9             | PARWOO1      | MN104962  | Parmoptila woodhousei |
| 10            | CYAOLI19     | MN104961  | Cyanomitra olivacea |
| 11            | STEGRA02     | MN104956  | Steidigillas gracilirostris |
| 12            | STEGRA03     | MN104955  | Steidigillas gracilirostris |
| 13            | BLESY02      | MN104960  | Bleda syndactylus |
| 14            | CRICHL01     | MN104958  | Criniger chloronotus |

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 truly distinct Trypanosoma SSU rRNA lineages. Numbers along branches correspond to node support from posterior probabilities. 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. litirosistris (t = -2.54, p = 0.01), P. icterusinus (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 (ctb 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 haemosporidan (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, Aedoeomyia) and wild mosquitoes of the genus Coquillettidia have been implicated in the transmission of different species of avian Plasmodium Bonneauad 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 simuliiids, 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-ALD11) *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* trypmastigotes 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*, *Leucozytozoon* and Nematode microfilariae 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 *Leucozytozoon* is underrepresented in the literature. This study has focused not only on *Plasmodium*, *Haemoproteus* and *Leucozytozoon* 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 Haemoporida 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. Elkwro. 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.

References

1. Brochet ALW, Vav Den Bossche VR, Jones H, et al. Illegal killing and taking of birds in Europe outside the Mediterranean: assessing the scope and scale of a complex issue. Bird Conservation. International. 2017; 1-31.
2. Oaks JL, Gilbert M, Virani MZ, et al. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature. 2004; 427: 630-633.
3. Ogada D, Shaw P, Beyers RL, et al. Another continental vulture crisis: Africa's vultures collapsing toward extinction. Conserv Lett. 2016; 9: 89-97.
4. Garbett R, Maude G, Hancock P, et al. Association between hunting and elevated blood lead levels in the critically endangered African white-backed vulture Gyps Africanus. Sci Total Environ. 2018; 630: 1654-1665.
5. Ferraz G, Nichols JD, Hines JE, et al. A large-scale deforestation experiment: effects of patch area and isolation on Amazon birds. Science. 2007; 315: 238-241.
6. FAO. State of the World’s Forests (FAO, 152 pp. Rome Italy: FAO. 2009.
7. Stouffer PC, Johnson EI, Bierregaard RO Jr, et al. Understory Bird Communities in Amazonian Rainforest Fragments: Species Turnover through 25 Years Post-Isolation in Recovering Landscapes. PLoS ONE. 2011; 6: e20543.
8. Valkiūnas G. Avian Malaria Parasites and Other Haemosporidia. CRC Press, Boca Raton Florida. 2005: 946.
9. Lutz HL, Hochachka WM, Engel JL, et al. Parasite Prevalence Corresponds to Host Life History in a Diverse Assemblage of Afrotropical Birds and Haemosporidian Parasites. PLoS ONE. 2015; 10: e0121254.
10. Clark NJ, Konstans W, Dimitar D, et al. Co-infections and environmental conditions drive the distributions of blood parasites in wild birds. Journal of Animal Ecology. 2016; 85: 1461-1470.
11. De Viache P, Greiner EC, Manteca X. Interspecific variability of prevalence in blood parasites of adult passerine birds during the breeding season in Alaska. Journal of Wildlife Diseases. 2001; 37: 28-35.
12. Read AF, Taylor LH. The ecology of genetically diverse infections. Science. 2001; 292: 1099-1102.
13. De Roode JC, Pansini R, Cheesman SJ, et al. Virulence and competitive ability in genetically diverse malaria infections. Proc Natl Acad Sci U.S.A. 2005; 102: 7624-7628.
14. Marzal A, Bensch S, Reviriegio M, et al. Effects of malaria double infection in birds: one plus one is not two. Journal of Evolutionary Biology. 2008; 21: 979-987.
15. Mosquera J, Adler FR. Evolution of virulence: A unified framework for coinfection and superinfection. Journal of Theoretical Biology. 1998; 195: 293-313.
16. Taylor LH, Mackinnon MJ, Read AF. Virulence of mixed-clone and single-clone infections of the rodent malaria Plasmodium chabaudi. Evolution. 1998; 52: 583-591.
17. Taylor LH, Welburn SC, Woolhouse MEJ. Theileria annulata: Virulence and transmission from single and mixed clone infections in cattle. Experimental Parasitology. 2002; 100: 186-195.
18. De Roode JC, Read AF, Chan BHK, et al. Rodent malaria parasites suffer from the presence of conspecific clones in three-clone Plasmodium chabaudi infections. Parasitology. 2003; 127: 411-418.
19. Chasar A, Loiseau C, Valkiūnas G, et al. Prevalence and diversity patterns of avian blood parasites in degraded African rainforest habitats. Molecular Ecology. 2009; 18: 4121-4133.
20. Bonneau C, Sepil I, Mla ÅB, et al. The prevalence of avian Plasmodium is higher in unfragmented tropical forests of Cameroon. Journal of Tropical Ecology. 2009; 25: 439.
21. De wasseige c, De marcken P, Bayol N, et al. The forest of the Congo Basin-State of the forest (2010) Publications Office of the European Union, Luxembourg. 2012.
22. Patz JA, Graczyk TK, Geller N, et al. Effects of environmental change on emerging parasitic diseases. International Journal of Parasitology. 2000; 30: 1395-1405.
23. Lettink M, Armstrong DP. An introduction to using mark-recapture analysis for monitoring threatened species. Department of Conservation Technical Series. 2003; 28: 5-32.
24. Wood MJ, Cosgrove CL, Wilkin TA, et al. Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, Cyanistes caeruleus. Molecular Ecology. 2007; 16: 3263-3273.
25. Loiseau C, Iezhova T, Valkiūnas G, et al. Spatial variation of haemosporidian parasite infection in African rainforest bird species. Journal of Parasitology. 2010; 96: 21-29.
26. Palinauskas V, Valkiūnas G, Bolshakov CV, et al. Plasmodium relictum (lineage SGS1) and Plasmodium ashfordi (lineage GRW2): the effects of the coinfection on experimentally infected passerine birds. Vet Parasitol. 2011; 127: 527-533.
27. Sehgal RNM, Buermann W, Harrigan RJ, et al. Spatially explicit predictions of blood parasites in a widely distributed African rainforestbirds. Proceedings of the Royal Society B. 2011; 278: 1025-1033.
28. Laurence SGW, Jones D, Westcott D, et al. Habitat fragmentation and ecological traits influence the prevalence of avian blood parasites in a Tropical Rainforest landscape. PLoS ONE. 2013; 8: e76227.
30. Valkiūnas G, Iezhova TA, Ravinder NMS. Deforestation does not affect the prevalence of a common trypanosome in African birds. Acta Tropica. 2016; 162: 222-228.
31. Tchoumbou MA, Mayi MPA, Malange ENF, et al. Effect of deforestation on the prevalence of avian haemosporidian parasites and mosquito abundance in a tropical rainforest of Cameroon. International Journal for Parasitology. 2019; 50: 63-73.
32. Oakgrove KS, Ryan JH, Claire L, et al. Distribution, diversity and drivers of blood-borne parasite co-infections in Alaskan bird populations. International Journal for Parasitology. 2014; 44: 717-727.
33. Sehgal RNM. Manifold habitat effects on the prevalence and diversity of avian blood parasites. International Journal for Parasitology: Parasites and Wildlife. 2015; 4: 421-430.
34. Juhl J, Permin A. The effect of *Plasmodium gallinaceum* on a challenge infection with *Ascaridia galli* in chickens. Veterinary Parasitology. 2002; 105: 11-19.
35. Williams NA, Bennett GF, Troncy PM. Avian hematozoa of some birds from Tchad. Journal of Wildlife Diseases. 1977; 13: 9-61.
36. Bennett GF, Blancou J, White EM, et al. Blood parasites of some birds from Senegal. Journal of Wildlife Diseases. 1978; 14: 67-73.
37. Kirkpatrick CE, Smith TB. Blood parasites of birds in Cameroon. Journal of Parasitology. 1988; 74: 1009-1013.
38. Sehgal RNM, Jones HI, Smith TB. Molecular evidence for host specificity of parasitic nematode microfilariae in some African rainforest birds. Molecular Ecology. 2005; 14: 3977-3988.
39. Mittermeier RA, Gil PR, Hoffman M, et al. Hotspots revisited: Earth's biologically richest and most threatened terrestrial ecoregions. CEMEX Mexico City. 2005.
40. Oates JF, Bergl RA, Linder JM. Africa’s Gulf of Guinea Forest: Biodiversity Patterns and Conservation Priorities. Advances in Applied Biodiversity Science 6. Conservation International Washington DC USA. 2004.
41. Sehgal RNM, Jones HI, Smith TB. Host specificity and incidence of *Trypanosoma* in some African rainforest birds: a molecular approach. Molecular Ecology. 2001; 10: 2319-2327.
42. Borrow N, Demey R. Birds of Western Africa. Second Edition. 2014; 592.
43. Sibley CG, Monroe BLJr. Distribution and Taxonomy of Birds of the World. Yale University Press, New Haven, Connecticut P1111. 1990.
44. Smith TB, Wayne R, Girman K, et al. A role of ecotone in generating rainforest biodiversity. Science. 1997; 276: 1855-1857.
45. Valkiūnas G, Iezhova TA, Krizankauskiene A, et al. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Journal of Parasitology. 2008; 94: 1395-1401.
46. Godfrey RD, Fedynich AM, Pence DB. Quantification of hematozoa in blood smears. Journal of Wild Diseases. 1987; 23: 558-565.
47. Valkiūnas G, Bensch S, Iezhova TA, et al. Nested cytochrome b polymerase chain reaction diagnostics underestimate mixed infections of avian blood haemosporidian parasites: microscopy is still essential. Journal of Parasitology. 2006; 92: 418-422.
62. Cellier-Holzem E, Esparza-Salas R, Garnier S, et al. Effect of repeated exposure to Plasmodium relictum (lineage SGS1) on infection dynamics in domestic canaries. International Journal of Parasitology. 2010; 40: 1447-1453.
63. Bichet C, Sorci G, Robert A, et al. Epidemiology of Plasmodium relictum infection in the house sparrow. Journal of Parasitology. 2014; 100: 59-65.
64. Gouagna LC, Bonnet S, Gounoue R, et al. Stage-specific effects of host plasma factors on the early sporogony of autologous Plasmodium falciparum isolates within Anopheles gambiae. Trop Med Int Health. 2004; 9: 937-948.
65. Svobodová M, Volf P, Votýpka J. Trypanosomes and haemosporidia in the buzzard (Buteo buteo) and sparrowhawk (Accipiter nisus): factors affecting the prevalence of parasites. Parasitological Research. 2015; 114: 551-560.
66. Soares L, Vincenzo A Ellis, Robert ER. Co-infections of haemosporidian and trypanosome parasites in a North American songbird. Cambridge University Press Parasitology. 2016; 143: 1930-1938.
67. Njabo KY, Cornel AJ, Sehgal RN, et al. Coquillettidia (Culicidae, Diptera) mosquitoes are natural vectors of avian malaria in Africa. Malaria Journal. 2009; 8: 193.
68. Schmid S, Dinkel A, Mackenstedt U, et al. Avian malaria on Madagascar: bird host and putative vector mosquitoes of different Plasmodium lineages. Parasite and Vectors. 2017; 10: 6.
69. Greiner EC, Bennett GF, White EM, et al. Distribution of the avian hematozoa of North America. Canadian Journal of Zoology. 1975; 53: 1762-1787.
70. Beaudoin RL, Applegate JE, David DE, et al. A model for the ecology of avian malaria. Journal of Wildlife Diseases. 1971; 7: 5-13.
71. Kucera J. Blood parasites of birds in Central Europe III Plasmodium and Central Europe. Folia Parasitologica. 1981; 28: 303-312.
72. Weatherhead PJ, Bennett GF. Ecology of red-winged blackbird parasitism by hematozoa Canadian. Journal of Zoology Revue Canadienne de Zoologie. 1991; 69: 2352-2359.
73. Schrader MS, Walters EL, James FC, et al. Seasonal prevalence of a haematozoan parasite of red-bellied woodpeckers (Melanerpes carolinus) and its association with host condition and overwinter survival. Auk. 2003; 120: 130-137.
74. Baker JR. Biology of the trypanosomes of birds. In: Lumsden, W.H.R., Evans, D.A. (Eds.), Biology of the Kinetoplastida, vol. 1. Academic Press London UK. 1976; 131-174.
75. Apanius V. Avian trypanosomes as models of hemolagellate evolution. Parasitology Today. 1991; 7: 87-90.
76. Bennett GF, Okia NO, Cameron MF. Avian hematozoa of some Ugandan birds. Journal of Wildlife Diseases. 1974; 10: 458-465.
77. Bennett GF, Herman CM. Blood parasites of some birds from Kenya, Tanzania and Zaire. Journal of Wildlife Diseases. 1977; 12: 59-65.