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Hemoparasites in a wild primate: Infection patterns suggest interaction of *Plasmodium* and *Babesia* in a lemur species☆

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ABSTRACT

Hemoparasites can cause serious morbidity in humans and animals and often involve wildlife reservoirs. Understanding patterns of hemoparasite infections in natural populations can therefore inform about emerging disease risks, especially in the light of climate change and human disruption of natural ecosystems. We investigated the effects of host age, sex, host group size and season on infection patterns of *Plasmodium* sp., *Babesia* sp. and filarial nematodes in a population of wild Malagasy primates, Verreaux's sifakas (*Propithecus verreauxi*), as well as the effects of these infections on hematological variables. We tested 45 blood samples from 36 individuals and identified two species of *Plasmodium*, one species of *Babesia* and two species of filarial nematodes. *Plasmodium* spp. and *Babesia* sp. infections showed opposite patterns of age-dependency, with babesiosis being prevalent among young animals, while older animals were infected with *Plasmodium* sp. In addition, *Babesia* sp. infection was a statistically significant negative predictor of *Plasmodium* sp. infection. These results suggest that *Plasmodium* and *Babesia* parasites may interact within the host, either through cross-immunity or via resource competition, so that *Plasmodium* infections can only establish after babesiosis has resolved. We found no effects of host sex, host group size and season on hemoparasite infections. Infections showed high prevalences and did not influence hematological variables. This preliminary evidence supports the impression that the hosts and parasites considered in this study appear to be well-adapted to each other, resulting in persistent infections with low pathogenic and probably low zoonotic potential. Our results illustrate the crucial role of biodiversity in host-parasite relationships, specifically how within-host pathogen diversity may regulate the abundance of parasites.

1. Introduction

Vector-borne hemoparasites, including the apicomplexan protozoa *Babesia* sp. and *Plasmodium* sp., trypanosomes and filarial nematodes, are important pathogens in humans and domestic animals, causing babesiosis, malaria, sleeping sickness, lymphatic filariasis and canine heartworm disease. Endemic as well as introduced hemoparasites may also impact health and fitness of wildlife (e.g. Custer and Pence, 1981; Atkinson et al., 2000; Garvin et al., 2003; Donahoe et al., 2015). Furthermore, babesiosis is an emerging zoonosis worldwide, with wildlife reservoirs playing a particular role in its epidemiology (Gray et al., 2010; Yabsley and Shock, 2013). Thus, characterizing hemoparasite infections in wildlife and understanding patterns of prevalence can potentially reveal emerging disease risks, especially in the light of ecological instabilities as a result of human encroachment into wildlife habitats (Daszak et al., 2001; Keesing et al., 2010) and climate change (Daszak et al., 2000; Barrett et al., 2013; Kronefeld et al., 2014). Wild primate populations are of particular interest in this context, because nonhuman primates have played a major role in the emergence of human diseases, including malaria, in the past (Wolfe et al., 2007; Pedersen and Davies, 2009; Pacheco et al., 2011).

Age, sex or seasonal effects may have an influence on exposure

☆ Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession number(s) LN869519 – LN869522.

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and susceptibility to infection with many parasites. Age effects have been demonstrated for *Plasmodium* sp. infections in chimpanzees and humans (Doolan et al., 2009; De Nys et al., 2013) and for *Babesia* sp. infections in dogs and cattle (Boozer and Macintire, 2003; Bock et al., 2004), with decreasing prevalence in older animals being most likely due to the development of an effective adaptive immune response (Frolich et al., 2012). Additionally, the innate immune system is an important controlling factor of apicomplexan parasite infectivity (Frolich et al., 2012) and variation in immune responsiveness may thus influence infection patterns.

Sex differences in hemoparasite infections have been found in several vertebrates, including higher prevalences in male penguins (Merkel et al., 2007), lizards (Schall et al., 2000) and lions (Sherman, 2010). These sex differences could either be due to physiological differences, e.g. immunosuppression caused by higher testosterone levels in males (Klein, 2004; Roberts et al., 2004), or due to differences in parasite exposure. For example, a sex-bias in vector feeding rates towards males was recently demonstrated in birds (Burkett-Cadena et al., 2014). Although hemoparasite infections have been investigated in several wild primate species (de Thoisy et al., 2001; Maamun et al., 2011; De Nys et al., 2013; Thurber et al., 2013), no sex-differences in prevalence have been reported so far.

Furthermore, it has been proposed that group-living may decrease the risk of infection with vector-borne pathogens by means of an encounter-dilution effect, analogous to a decrease of predation risk (Freeland, 1976; Mooring and Hart, 1992; Kappeler et al., 2015). Empirical evidence for this effect is controversial, however. Krebs et al. (2014) recently demonstrated that sentinel hosts caged inside large roosts of American robins seroconverted to West Nile Virus more slowly than those held outside of roosts, suggesting that exposure of individual hosts can indeed be reduced through group-formation. However, larger groups may also be more conspicuous, and thus attract more vectors. In a comparative study, colonially-breeding bird species were shown to experience both higher prevalences and higher species diversity of blood parasites than solitarily breeding species (Tella, 2002). Two studies on Neotropical primates found that prevalence of *Plasmodium* sp. increases with group size (Davies et al., 1991; Nunn and Heymann, 2005), but studies on the effect of within-species variability of group size on hemoparasite infections in primates are lacking.

Furthermore, infections with vector-borne parasites are likely to covary with environmental conditions (Altizer et al., 2006). Rainfall and temperature affect mosquito abundance, biting rates and parasite development within mosquitoes (Altizer et al., 2006; Galardo et al., 2009; Mohammed and Chadee, 2011). Additionally, dry, cold conditions as well as frequent temperature fluctuations can reduce the abundance of host-seeking ticks (Sutherst and Bourne, 2006; Swai et al., 2006; Herrmann and Gern, 2013). Despite this seasonality in vector biology, empirical evidence regarding seasonal variation in host infection rates is contradictory and mainly limited to malaria in humans (Smith et al., 1993; Koram et al., 2003; Mabaso et al., 2007). If transmission results in persistent infections, little seasonal variation in prevalence can be expected (Govender et al., 2011).

Finally, in natural systems co-infections with multiple parasites are common, potentially resulting in complex interspecific interactions. It has been shown that parasite community interactions may explain more variation in infection risk than the effects associated with host and environmental factors. For example, in field voles (*Microtus agrestis*) chronic infection with *Babesia microti* reduces susceptibility to *Bartonella* spp. bacteria by 85% as compared to uninfected individuals (Telfer et al., 2010). Parasites may directly affect the fitness of co-infecting species through interference competition, they may compete for the same resources within a host, or affect each other's abundance via interaction with the host's immune system (Pedersen and Fenton, 2006). Cross-immunity between co-infecting parasite species might limit prevalence at the population level, whereas parasite-induced immunosuppression may lead to synergistic effects (Cox, 2001; Telfer et al., 2008).

We present the first study which systematically characterizes infections with several co-occurring hemoparasites in a population of Malagasy primates, Verreaux's sifakas (*Propithecus verreauxi*). Madagascar faces acute risks of species extinctions as well as disease emergence in humans and wildlife due to intense human disruption of natural ecosystems (Harper et al., 2007; Junge, 2007; Barrett et al., 2013; Ratsimbazafy et al., 2013; Schwitzer et al., 2014). Verreaux's sifakas are diurnal, sexually monomorphic lemurs which live in multi-male multi-female groups of varying size in seasonal habitats, in which mosquitoes are virtually absent for several months of the year, in western and southern Madagascar (Kappeler and Fichtel, 2012). The life expectancy of sifakas in the wild can exceed 20 years (Richard et al., 2002; Kappeler and Fichtel, 2012). We used samples collected from members of all age classes from 10 adjacent groups ranging in size from 2 to 7 individuals during annual captures to detect infections with *Plasmodium* sp., *Babesia* sp. and filarial nematodes using a combination of a PCR-based approach and microscopical examination of blood smears. These parasites have previously been reported to occur in blood samples of sifakas (Uilenberg et al., 1972; Junge and Louis, 2005; Duval et al., 2010; Pacheco et al., 2011; Rasambainarivo et al., 2014), but systematic investigations of infection patterns are lacking. The potential vectors for *Plasmodium* spp. and *Babesia* spp., anopheline mosquitos and haemaphysaline ticks, are present in the study region (Davidson, 1966; Rodriguez et al., 2012), whereas the vectors for filarial parasites of lemurs are unknown (Irwin and Raharison, 2009).

To illuminate natural drivers of infection patterns, we tested the influence of host age and sex, host group size and seasonality on individual infection status and hemoparasite species richness in our study population. To assess possible health consequences, we also tested the influence of these infections on packed cell volume, to assess possible anemia, and total plasma protein as well as the neutrophil-lymphocyte ratio, which are usually increased during inflammatory processes (Thrall et al., 2006). Based on results of previous studies with other vertebrates, we predicted that infection probabilities would decrease with age and that males would harbor more hemoparasites than females. Furthermore, we predicted that group size would negatively affect the probability of testing positive for each hemoparasite, as well as individual hemoparasite species richness, in case of an encounter-dilution effect. Alternatively, we predicted that group size would have a positive effect on these measures if larger groups attract more vectors. We also expected to find lower prevalences of hemoparasite infections during the dry season than during the hot, wet season.

2. Materials and methods

2.1. Sample collection

The study was carried out in Kirindy Forest, Western Madagascar, located at approximately 44°39′E, 20°03′S. The study area is part of a field site operated by the German Primate Center (DPZ) since 1993 and is situated within a forestry concession managed by the Centre National de Formation, d'Etudes et de Recherche en Environnement et Foresterie (CNFEREF). Kirindy Forest is a dry deciduous forest and subject to pronounced seasonality, with a dry season from April to October and a hot, wet

season from November to March (Kappeler and Fichtel, 2012). As part of an ongoing long-term study (Kappeler and Fichtel, 2012), several social groups of Verreaux's sifakas have been habituated to human observers and individually marked with microchips and unique collars.

A total of 45 blood samples were taken from 36 individual Verreaux's sifakas belonging to 10 social groups during routine immobilization procedures in April 2013, August 2013, March 2014 and April 2014. Group sizes ranged from 2 to 7 individuals, and a minimum of 43% of individuals were sampled from each group. Seven animals were repeatedly sampled at intervals of 4–5 months, resulting in a maximum of 3 samples per individual (Table 1). Blood was collected from the femoral vein into anticoagulant EDTA tubes. Packed cell volume was determined by centrifuging EDTA blood in a microhematocrit capillary using a Sigma 1–14 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) and total plasma protein was estimated using a hand-held refractometer. Two to 3 blood smears were prepared per individual, air dried, stained (Diff Quick stain, Eberhard Lehmann GmbH, Berlin, Germany) and preserved with mounting medium (Eukitt, FLUKA Analytics, Sigma–Aldrich Chemie Gmbh, Munich, Germany) and a cover slip. Blood smears were scanned for the presence of hemoparasites and used for a differential white blood cell count to assess the percentages of the following leukocyte categories: Neutrophils, banded neutrophils, lymphocytes, monocytes, eosinophils and basophils. Photographs were taken with a Zeiss AxioCam ERc 5s fitted to a Zeiss Primo Star microscope (Carl Zeiss AG, Oberkochen, Germany). Measurements were made using the Zeiss Zen lite 2012 software (Carl Zeiss AG) after calibration with a stage micrometer. An aliquot of blood was mixed with the same amount of RNAlater and frozen at −20 °C until shipment to Germany and further analysis.

All necessary research permits were obtained from the Malagasy and German authorities and the study was approved by the Ethics Committee of the German Primate Center.

2.2. DNA extraction, PCRs and sequence analyses

DNA was extracted from RNAlater-preserved blood samples using the QIAmp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To test for the presence of *Plasmodium* spp. in blood samples, a

Table 1
Details of sampling and infection patterns. Individuals are ordered by age. X denotes a positive, while – denotes a negative sample.

ID	Age (years)	Sex	Group	Group size	Sampling date	Season	Plasmodium	Babesia	Onchocercidae	
									Species 1	Species 2
Bet	1	m	C	4	11-Apr-14	wet	–	–	–	–
Buf	1	m	F1	6	26-Mar-13	wet	–	X	–	–
Col	1	m	F	5	25-Mar-13	wet	–	–	–	–
Dav	1	m	H	4	11-Apr-14	wet	–	X	–	–
Fre	1	m	F	6	11-Apr-14	wet	–	X	–	–
Gay	1	f	L	4	12-Apr-14	wet	–	X	–	–
Kam	1	f	J	7	26-Mar-13	wet	–	X	–	–
Man	1	m	C	4	25-Mar-13	wet	–	X	–	–
Win	1	m	G	6	11-Apr-14	wet	–	X	–	–
Yon	1	m	J	7	11-Apr-14	wet	–	X	–	–
Zwo	1	m	S3	7	25-Mar-13	wet	–	X	–	–
Gan	4	m	L	4	23-Apr-14	wet	–	X	X	–
Ich	4	m	J	6	20-Aug-13	dry	–	X	–	–
Mor	4	m	H	4	19-Mar-14	wet	–	X	X	X
Aug	5	f	E	4	10-Sep-13	dry	–	X	X	–
Aug	6	f	E	4	12-Mar-14	wet	–	X	X	–
Hel	5	f	F	5	26-Mar-13	wet	–	–	X	X
Hon	5	m	G	4	21-Aug-13	dry	–	–	–	X
Nov	5	f	G	6	11-Apr-14	wet	–	X	X	–
Ven	5	m	S3	6	13-Apr-14	wet	–	–	–	–
Bor	6	m	S3	6	13-Apr-14	wet	X	–	X	X
Kan	6	m	F1	3	14-Apr-14	wet	–	X	–	X
Kyu	6	m	G	6	11-Apr-14	wet	X	X	X	–
Lin	6	m	F1	5	20-Aug-13	dry	–	X	–	–
Sen	6	m	J	6	21-Aug-13	dry	X	–	–	X
Sen	7	m	J	7	13-Mar-14	wet	X	X	X	X
Ten	7	m	C	4	11-Apr-14	wet	–	X	X	–
Zur	9	m	G	5	16-Apr-13	wet	–	–	X	X
Zur	9	m	G	4	21-Aug-13	dry	X	–	X	X
Zur	10	m	G	5	13-Mar-14	wet	X	–	X	X
Goa	10	f	L	3	26-Mar-13	wet	–	–	X	X
Mel	10	m	E	6	16-Apr-13	wet	X	–	X	–
Mel	10	m	E	5	20-Aug-13	dry	X	–	X	X
Sis	11	f	E	6	28-Mar-13	wet	X	X	X	X
Fra	12	m	F	5	21-Aug-13	dry	X	–	X	–
Fra	12	m	F	6	12-Mar-14	wet	X	–	X	–
Boc	13	m	C	4	20-Aug-13	dry	X	–	X	X
Boc	14	m	C	4	13-Mar-14	wet	X	–	X	X
Sav	14	f	F1	6	26-Mar-13	wet	X	–	–	–
Swa	14	f	S2	2	14-Apr-14	wet	X	–	X	–
Maf	16	m	L	3	22-Aug-13	dry	X	–	X	–
Sap	19	m	H	3	16-Apr-13	wet	X	–	X	–
Sap	19	m	H	3	22-Aug-13	dry	X	–	–	–
Sap	20	m	H	4	11-Apr-14	wet	X	–	X	–
Yok	21	f	J	7	11-Apr-14	wet	X	–	–	X

semi-nested PCR was carried out targeting an approximately 1000 bp long fragment of the parasite's cytochrome b gene. In the first amplification round, primers P.sp.cytB F1 (5′-TGC CTA GAC GTA TTC CTG ATT ATC CAG; Kaiser et al. (2010)) and P.sp.cytB R1 (5′- CTT GTG GTA ATT GAC ATC CWA TCC; Kaiser et al. (2010)) were used, followed by P.sp.cytB F2 (5′-ATT GGD TCA ACW ATG ACT TTA TTT GG) and P.sp.cytB R1 in the second round. The 25 μl reaction mixture contained 1 μl of DNA-extract or PCR-product (diluted 1:40) from the first round, respectively, 2.5 μl 10x PCR buffer (Invitrogen, Karlsruhe, Germany), 2 μl of 50 mM $MgCl_2$ (Invitrogen, Karlsruhe, Germany), 2 μl of 2.5 mM deoxynucleotide triphosphates (Thermo Scientific Fermentas, St. Leon-Rot, Germany), 0.5 μl of each primer (10 μM) and 0.2 μl of Platinum Taq polymerase (Invitrogen, Karlsruhe, Germany). The thermal profile was the same for both rounds, with an initial denaturation step at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 58 °C for 45 s and 72 °C for 60 s, and a final elongation step at 72 °C for 10 min.

We tested for *Babesia* spp. using primers BJ1 (5′-GTC TTG TAA TTG GAA TGA TGG-3′) and BN2 (5′-TAG TTT ATG GTT AGG ACT ACG-3′), targeting a 500 bp long fragment of the 18S rRNA gene (Casati et al., 2006). We used 5 μl of DNA-extract in a 25 μl reaction mixture containing the same quantities of reagents as stated above. The thermal profile consisted of an initial denaturation step at 94 °C for 10 min followed by 40 cycles of 94 °C for 60 s, 55 °C for 60 s and 72 °C for 2 min, and a final elongation step at 72 °C for 5 min.

To generate sequences for microfilaria observed in blood smears, we employed a nested PCR targeting an approximately 900 bp long fragment spanning part of the 18S rRNA gene, the internal transcribed spacer 1 (ITS1) and part of the 5.8S rRNA gene. Primers used were NF1 (5′-GGT GGT GCA TGG CCG TTC TTA GTT-3′) (Porazinska et al., 2009) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Gasser et al., 1993; Chilton et al., 2003) in the first round and a modification of ITS1-F (5′- TTG ATT ACG TCC CTG CCC-3′) (Vrain et al., 1992; Bisset et al., 2014) and the filaria-specific Di5.8S-R (5′-ACC CTC AAC CAG ACG TAC-3′) (Nuchprayoon et al., 2003, 2005) in the second round. The 25 μl reaction mixture contained 5 μl of DNA-extract in the first round and 1 μl PCR-product (diluted 1:40) in the second round, and the same quantities of reagents as in the other PCRs. The thermal profile of the first round consisted of an initial denaturation step at 95 °C for 5 min followed by 35 cycles of 94 °C for 60 s, 58 °C for 30 s and 72 °C for 60 s, and a final elongation step at 72 °C for 10 min. The thermal profile of the second round consisted of an initial denaturation step at 94 °C for 10 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, and a final elongation step at 72 °C for 10 min.

PCRs were run in a FlexCycler thermal cycler (Analytic Jena, Jena, Germany) and amplification products were detected by electrophoresis on 1.5% agarose gels. Positive and negative controls were included in all PCRs. PCR products of the corresponding size were purified from 2% agarose gels using the JETQUICK Gel Extraction Spin Kit (Genomed, Löhne, Germany) and Sanger sequencing of both strands was performed by Seqlab Sequence Laboratories Göttingen GmbH (Göttingen, Germany). Sequences were analysed using Geneious v6.1.6 (Biomatters Ltd., Auckland, New Zealand) and compared to publicly available sequences using BLAST (Altschul et al., 1990). New sequences were deposited in the EMBL Nucleotide Sequence Database (Kulikova et al., 2004), under accession numbers LN869519 – LN869522.

For *Plasmodium* sequences, we also performed phylogenetic analyses. The two unique sequences identified in this study were put together with the representative sequences selected by Pacheco et al. (2011), which includes all sequences derived from lemur *Plasmodium* available to this date. We used jModelTest v2.1.4 to identify the model of nucleotide substitution with the best fit to the data (GTR + I + G_4; (Darriba et al., 2012)). We then reconstructed a maximum likelihood tree under this model using PhyML v3 (Guindon et al., 2010), as implemented on the PhyML webserver (Guindon et al., 2005). Branch robustness was assessed through non-parametric bootstrapping (500 bootstrapped pseudo-replicates; Bp). Patristic distances were calculated using Geneious v6.1.6 (Biomatters Ltd.).

2.3. Statistical analyses

We used generalized linear-mixed models (GLMMs) with binomial error structure and logit link function to analyse which factors influenced the probability of testing positive for each parasite species. We included sex, age, season (wet or dry) and size of the animal's social group as fixed effects and individual identity nested in group as a random effect. Furthermore, we ran a GLMM with Poisson error structure and log link function to test influences on hemoparasite species richness, including the same fixed and random effects. Based on results of the GLMMs for *Plasmodium* and *Babesia* infections, we used another GLMM with binomial error structure to test whether *Babesia* infection status had an effect on *Plasmodium* infection, again controlling for individual identity nested in group. To assess potential health impacts, we first tested whether age affected hematology values, using Spearman rank correlation, and then used linear mixed models (LMMs) to test influences of infection with each hemoparasite and hemoparasite species richness on log-transformed packed cell volume, total plasma protein and the log-transformed neutrophil: lymphocyte ratio, controlling for animal age and individual identity nested in group as a random effect. We compared each full model to a null model comprising only the random effect in a likelihood ratio test using the R-function ANOVA with the argument 'test' set to 'Chisq'. Statistical significance was inferred if the specific P-value of the factor as well as the P-value of the likelihood ratio test were <0.05.

3. Results

3.1. Plasmodium infections

Thirteen of 36 individuals tested PCR-positive for *Plasmodium* spp. infection. Seven individuals were tested more than once, and repeated sampling at different times of the year almost always gave identical results for these animals. Only results for one animal (age 9) switched from negative to positive during the study period. Population prevalence is thus estimated at 36%. We did not observe any parasite stages in Diff-quick®-stained blood smears.

Only two unique sequences were amplified in this study, which showed 95–100% sequence identity to publicly available *Plasmodium* sequences. Most sequences could be unambiguously assigned to *Plasmodium* sp. LLD-2010 which has previously been found in Verreaux's sifakas (Duval et al., 2010). Two animals carried a different parasite (EMBL accession no.: LN869522), which unambiguously nested within the clade of malaria parasites infecting lemurs (Bp 100; Fig. 1). This parasite appeared as most closely related to *Plasmodium* sp. C-MAP-2012 (which has been found in *Hapalemur griseus* (Pacheco et al., 2011)), although the statistical support for this association was moderate (Bp 59; Fig. 1). The patristic distance between these two sequences was 0.21 substitution per site ($s.s^{-1}$), which is both higher than the 0.17 s s^{-1} observed between the two most closely related lemur parasite sequences known to date (*Plasmodium* sp. B-MAP.2012 and E-MAP-2011) and higher than the maximum 0.14 s s^{-1} observed between *P. reichenowii* (clade C1, infecting chimpanzees) and *P. praefalciparum* sequences (clade G1, infecting gorillas; Rayner et al., 2011). Coinfections with both lemur malaria parasites were not observed.

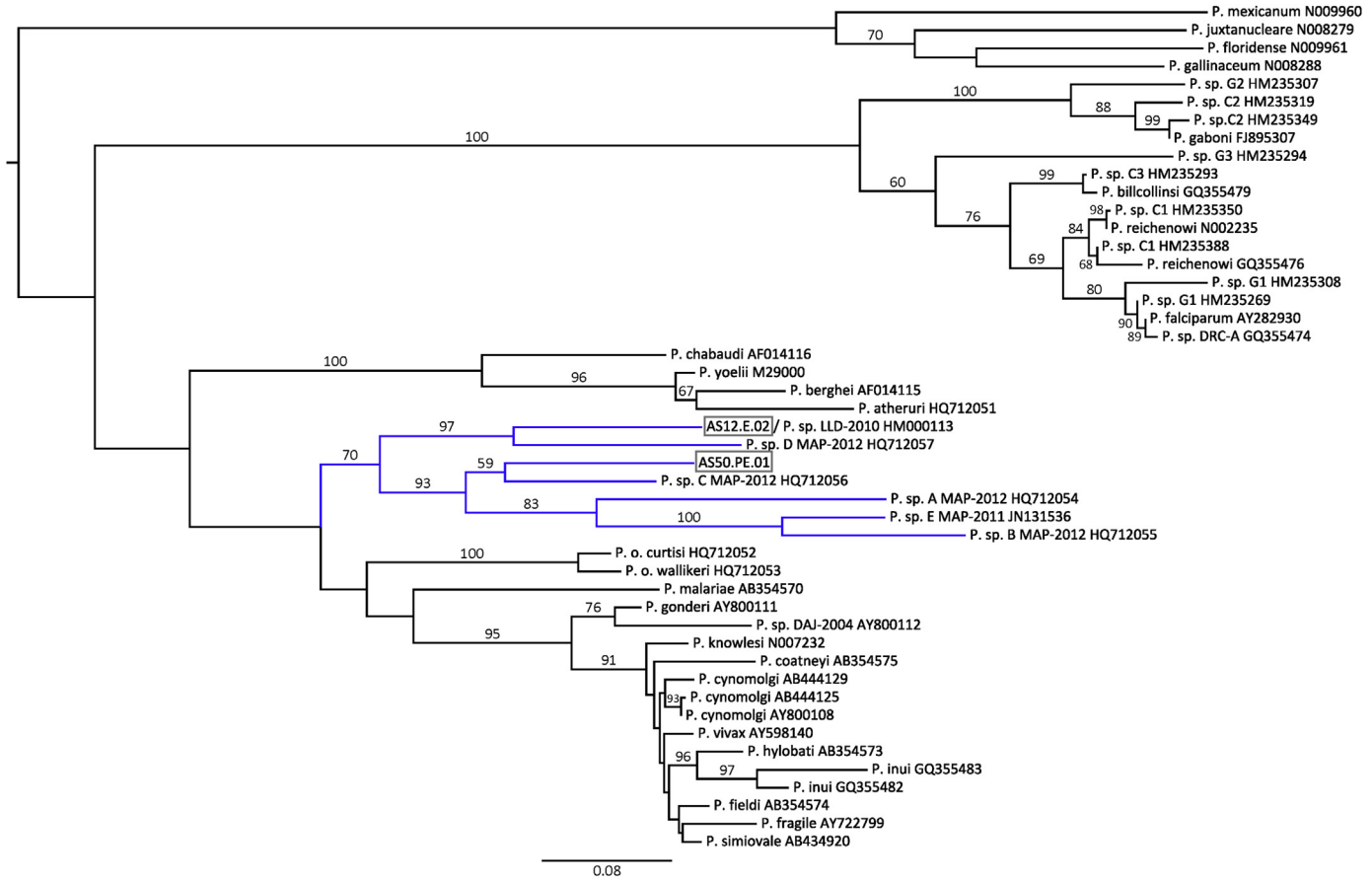

Fig. 1. Maximum likelihood tree of malaria parasite cytochrome b sequences (P. = Plasmodium). The clade formed by lemur malaria parasites is blue. The two sequences detected in this study are highlighted with grey rectangles. Bootstrap values are reported above branches when >50. The scale is in substitution per site.

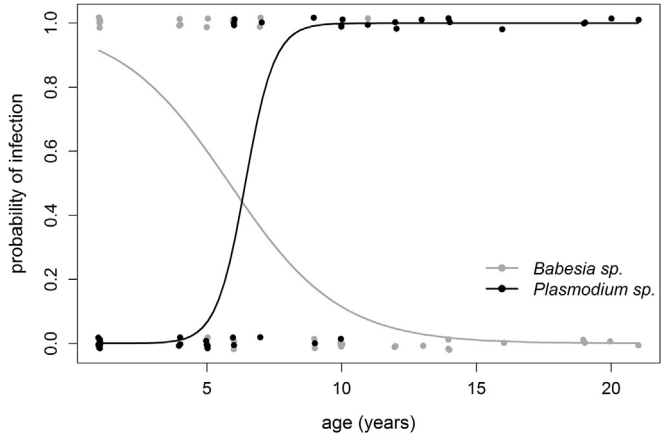

Fig. 2. Age-dependence of *Babesia* sp. infections (grey) and *Plasmodium* sp. infections (black). The lines represent the predicted values according to the two different GLMMs.

Age seemed to affect *Plasmodium* spp. test outcome, with older animals being more likely to test positive (Fig. 2). In fact, none of the animals up to the age of 5 years were infected (n = 18), 50% of animals between age 6 and 10 were infected (n = 10) and all animals over the age of 10 tested positive (n = 8). However, in the GLMM including age as well as sex, season and group size as fixed factors, age only tended to have a statistically significant effect (Table 2). Sex, season and group size had no significant effect on *Plasmodium* spp. infection status.

Table 2
Results of GLMM testing the influence of different predictor variables on the probability of being infected with *Plasmodium* spp. Significant P-values are printed in bold. Likelihood ratio test comparing the full model to a null model containing only the random effect: $\chi^2 = 37.2$, df = 4, **P < 0.001**. Significant P-values are printed in bold.

Term	Estimate	Standard error	z	P
Intercept	−28.59	16.76	−1.71	0.088
Sex	0.06	2.99	0.02	0.98
Age	1.86	0.98	1.89	0.059
Group size	3.3	2.3	1.43	0.153
Season	−2.68	2.41	−1.11	0.267

3.2. *Babesia* infections and potential *Plasmodium* – *Babesia* interaction

Prevalence of *Babesia* infections was 55.6%. Again, test results for only 1 individual (age 7) changed from negative to positive during

Table 3
Results of GLMM testing the influence of different predictor variables on the probability of being infected with *Babesia* sp. Significant P-values are printed in bold. Likelihood ratio test comparing the full model to a null model containing only the random effect: $\chi^2 = 23.7$, df = 4, **P < 0.001**.

Term	Estimate	Standard error	z	P
Intercept	1.08	2.72	0.4	0.692
Sex	−1.51	1.29	−1.16	0.244
Age	−0.49	0.22	−2.2	**0.028**
Group size	0.35	0.5	0.71	0.479
Season	1.7	1.49	1.14	0.254

the study period. The *Babesia* sequence amplified (EMBL accession no. LN869519) was the same in all samples and showed 94% identity to different *Babesia canis* isolates. We did not microscopically observe any *Babesia* parasite stages in Diff-quick®-stained blood smears.

Only age significantly affected *Babesia* sp. test outcome, but contrary to the *Plasmodium* sp. infections, young animals were more likely to test positive (Table 3, Fig. 2). Only 3 animals (6, 7 and 11 years old) were co-infected with *Plasmodium* sp. and *Babesia* sp. Based on this complementary age pattern, the low number of co-infections, and because neither age, sex, season nor group size influenced *Plasmodium* spp. infections, we tested *Babesia* infection status as a predictor of *Plasmodium* infection, and found a highly significant negative effect (Table 4).

3.3. Filarial nematode infections

Two different filarial nematode species could be differentiated morphologically as well as genetically. Two to 3 blood smears per sample were scanned for microfilaria and on average 4.2 microfilaria were measured per sample. Microfilaria of species 1 were 268 ± 10.1 μm long with the nerve ring located at 53 ± 4.5 μm from the anterior end. Microfilaria of species 2 were considerably shorter, 222 ± 18.6 μm, with the nerve ring located at 43 ± 5.4 μm.

Sequencing of PCR products confirmed the presence of 2 different species. Species 1 produced a shorter band of about 850 bp and species 2 produced a longer band of about 900 bp. The bands could be separated out using 2% agarose gels, and individually sequenced. The sequences (EMBL accession no. LN869520 and LN869521) showed 99–100% sequence identity to different filarial nematodes such as *Mansonella* sp., *Dirofilaria* sp. and *Onchocerca* sp. in the conserved 18S and 5.8S regions, but only 84–90% similarity to other filarial nematodes in the variable ITS1 region.

In 33 of 45 samples, the PCR results exactly confirmed the morphological diagnosis (negative sample, presence of one or both species). In 7 cases, a species was morphologically identified but not detected by PCR, while in 5 cases an infection was revealed by PCR only. Thus, neither method seems to be 100% sensitive. For statistical analyses, we considered an infection present if it was detected by one of the methods.

Prevalence of species 1 was 50%, while species 2 was found in 33.3% of animals. Nine animals (25%) were co-infected with both species. No filarial nematode infections were detected in animals under the age of one year. Probability of infection with species 1 significantly increased with age (Table 5). We did not find any significant influences on infection with the second species (Table 5).

3.4. Hemoparasite species richness

We tested whether host age, sex, season or group size affected the number of hemoparasite species found in each individual, but we did not find any statistically significant influences (Table 6).

Table 4
Results of GLMM testing the influence of *Babesia* sp. infection on the probability of being infected with *Plasmodium* spp. Significant P-values are printed in bold. Likelihood ratio test comparing the full model to a null model containing only the random effect: $\chi^2 = 9.21$, df = 1, **P = 0.002**.

Term	Estimate	Standard error	z	P
Intercept	9.14	2.89	3.17	**0.002**
Babesia infection	−19.52	5.16	−3.78	**<0.001**

Table 5
Results of GLMMs testing the influence of different predictor variables on the probability of being infected with two different filarial nematodes (family Onchocercidae). Significant P-values are printed in bold. Likelihood ratio tests comparing full models to null models containing only the random effect: Onchocercidae sp. 1: $\chi^2 = 12.07$, df = 4, **P = 0.016;** Onchocercidae sp. 2: $\chi^2 = 1.63$, df = 4, P = 0.802.

Term	Estimate	Standard error	z	P
Onchocercidae sp. 1				
Intercept	−0.67	2.4	−0.28	0.78
Sex	0.02	0.97	0.03	0.98
Age	0.24	0.1	2.48	**0.013**
Group size	−0.38	0.39	−0.96	0.338
Season	1.36	1.06	1.28	0.202
Onchocercidae sp. 2				
Intercept	−3.88	10.88	−0.36	0.721
Sex	−0.25	4.41	−0.06	0.956
Age	−0.14	0.43	−0.33	0.739
Group size	−0.69	1.84	−0.38	0.707
Season	−3.68	4.59	−0.8	0.422

Table 6
Results of GLMM testing the influence of different predictor variables on hemoparasite species richness. Likelihood ratio test comparing the full model to a null model containing only the random effect: $\chi^2 = 3.7$, df = 4, P = 0.45.

Term	Estimate	Standard error	z	P
Intercept	0.1	0.59	0.18	0.859
Sex	−0.03	0.25	−0.13	0.897
Age	0.04	0.02	1.92	0.054
Group size	0.04	0.09	0.45	0.65
Season	0.07	0.26	0.27	0.79

3.5. Hematologic values

Hematology results are summarized in Table 7 Packed cell volume and total plasma protein were significantly positively correlated with the animals' age (Spearman rank correlation, N = 44, ρ = 0.44, P = 0.003, and N = 43, ρ = 0.4, P = 0.008, respectively) and the ratio of neutrophils to lymphocytes tended to be positively correlated with age (Spearman rank correlation, N = 45, ρ = 0.26, P = 0.08). Controlling for age, we did not find any significant influences of infection with any single parasite species on packed cell volume (PCV; Table 8), and hemoparasite richness also did not influence PCV (Table 9).

Likewise, total plasma protein (TP) was neither significantly influenced by infection with any single parasite species (Table 8), nor by hemoparasite species richness (Table 9).

The ratio of neutrophils to lymphocytes (NLR) was 0.47 ± 0.22 (range: 0.15–1.25). No single infection influenced log-transformed NLR values, (Table 8), and hemoparasite richness was also not a significant predictor (Table 9). The percentage of eosinophils was 0.96 ± 0.91 (range: 0–4.5), which is in the range of published values for *Propithecus* sp. (Bergeron and Buettner-Janusch, 1970; Junge and Louis, 2005; Irwin et al., 2010; Rasambainarivo et al., 2014). We thus concluded that no eosinophilia was present.

4. Discussion

We identified 5 putative species of hemoparasites in Verreaux's sifakas in Kirindy Forest: 2 species of malaria parasites, only one of which has been previously identified in Verreaux's sifakas by means of PCR (Duval et al., 2010), the other one likely standing for a yet undetected and undescribed species; one species of *Babesia*; and 2 species of filarial nematodes. As sequences of *Babesia* parasites and filarial nematodes from Madagascar are not publicly available to date, we could not assign species names to our

Table 7
Hematology results for Verreaux's sifakas captured at Kirindy Forest, Madagascar.

	All animals (N = 45)			Adults (N = 31)			1 – 4 year olds (N = 14)		
	Mean	Standard deviation	Min – Max	Mean	Standard deviation	Min – Max	Mean	Standard deviation	Min – Max
Packed cell volume (%)	38.05	3.58	29–47	39.4	2.74	35–47	35.14	3.44	29–43
Total plasma protein (g/dl)	6.68	0.49	5.4–7.8	6.83	0.38	6.2–7.6	6.39	0.54	5.4–7.4
Neutrophilic granulocytes, segmented (%)	27.64	8.88	14–49	28.88	7.78	17.5–49	25.16	9.98	12.5–40
Neutrophilic granulocytes, banded (%)	0.33	0.49	0–2	0.41	0.53	0–2	0.14	0.29	0–1
Lymphocytes (%)	63.37	9.95	39.5–81.5	62.37	8.51	39.5–76.5	65.72	11.66	49–81.5
Monocytes (%)	7.77	4.75	1.5–20.67	7.36	4.37	1.5–20.3	8.35	5.27	1.5–20.67
Eosinophilic granulocytes (%)	0.96	0.91	0–4.5	1.07	0.91	0–4.5	1.06	0.87	0–2.5
Basophilic granulocytes (%)	0.05	0.18	0–0.5	0.05	0.19	0–1	0.06	0.14	0–0.5

Table 8
Results of LMMs testing the influence of infection with each parasite species on hematology results. Packed cell volume and the neutrophil: lymphocyte ratio were log-transformed. Significant P-values are printed in bold. Likelihood ratio tests comparing full models to null models containing only the random effect: PCV: $\chi^2 = 7.9$, df = 5, P = 0.16; TP: $\chi^2 = 9.3$, df = 5, P = 0.1; NLR: $\chi^2 = 7.6$, df = 5, P = 0.18.

Term	Estimate	Standard error	df	t	P
Packed cell volume					
Intercept	3.55	0.04	33.45	85.86	**<0.001**
Plasmodium	0.004	0.04	28.54	0.09	0.929
Babesia	0.04	0.04	35.34	1.05	0.303
Onchocercidae 1	0.01	0.03	26.95	0.47	0.64
Onchocercidae 2	−0.02	0.03	33.91	−0.53	0.603
Age	0.008	0.005	36.93	1.77	0.085
Total plasma protein					
Intercept	6.6	0.21	32.3	31.05	**<0.001**
Plasmodium	0.09	0.23	32.31	0.39	0.698
Babesia	−0.22	0.19	36.07	−1.15	0.258
Onchocercidae 1	0.27	0.16	33.64	1.68	0.101
Onchocercidae 2	−0.02	0.17	32.02	−0.11	0.911
Age	0.002	0.02	29.52	0.11	0.912
Neutrophil: lymphocyte ratio					
Intercept	−1.01	0.21	34.87	−4.7	**<0.001**
Plasmodium	0.15	0.19	32.31	0.76	0.452
Babesia	−0.05	0.18	36.23	−0.29	0.776
Onchocercidae 1	−0.02	0.16	38.97	−0.15	0.885
Onchocercidae 2	−0.03	0.15	27.77	−0.22	0.829
Age	0.02	0.02	26.56	0.92	0.366

sequences. Morphological descriptions of microfilaria found in lemurs were ambiguous (Irwin and Raharison, 2009). Of the 4 species described in lemurs, which all belong to the family Onchocercidae, species 1 could either be *Dipetalonema petteri* or *Protofilaria furcata*, while measurements for species 2 fall into the range described for *Paulianfilaria pauliani*. We did not recover any adult worms, as dissections of animals were not possible in this study, and the insufficient quality of blood smears did not allow us to use further microfilaria characteristics for identification. However, genetic analyses confirmed the presence of two different filarial nematode species in the population.

We found high prevalence of infection with *Babesia* sp. in wild Verreaux's sifakas under the age of 5, while infections with *Plasmodium* sp. were absent from this age class, although most, but not all individuals of this age class were sampled during the wet season, when mosquitoes are abundant. This is unusual, as it has been found in humans and chimpanzees that younger animals usually have higher *Plasmodium* sp. prevalences and that prevalence decreases with age, possibly due to naturally acquired immunity (Doolan et al., 2009; De Nys et al., 2013). The age-infection patterns we observed give no indication of age-related immunity to Plasmodium sp. in Verreaux's sifakas. In our study, animals older than approximately 10 years showed 100% *Plasmodium* sp. prevalence, but no *Babesia* sp. infections. Only 3 animals (6, 7 and 11 years old) were co-infected with both parasites, and *Babesia* sp. infection was a statistically significant negative predictor for *Plasmodium* sp. infection.

Juveniles under the age of one year were never PCR-positive for filariasis, nor were microfilaria observed in their blood-smears. However, we cannot exclude the possibility that these animals were already infected, as the prepatent period of filarial nematodes, i.e. the period from infection to appearance of reproductive parasite stages in the blood, may span several months (Bowman and Georgi, 2009). Therefore, the result that age significantly affects filarial nematode infections should be treated with caution.

An additional caveat concerning filarial nematode infections should be kept in mind. Neither PCR nor microscopic examinations of blood smears seem to be sensitive enough to reliably detect filarial nematodes infections in all cases, so we combined the results of both methods. In cases of low parasitemia, PCRs produced some false-negatives although single microfilaria were identified in

Table 9
Results of LMMs testing the influence of hemoparasite species richness on hematology results. Packed cell volume and the neutrophil: lymphocyte ratio were log-transformed. Significant P-values are printed in bold. Likelihood ratio tests comparing full models to null models containing only the random effect: PCV: $\chi^2 = 6.06$, df = 2, P = 0.05; TP: $\chi^2 = 6.1$, df = 2, **P = 0.047**, $\chi^2 = 6.7$, df = 2, **P = 0.036**.

Term	Estimate	Standard error	df	t	P
Packed cell volume (PCV)					
Intercept	3.57	0.03	36.12	115.45	**<0.001**
Hemoparasite species richness	0.01	0.02	33.12	0.75	0.46
Age	0.006	0.003	30.43	1.87	0.072
Total plasma protein (TP)					
Intercept	6.39	0.17	30.81	38.26	**<0.001**
Hemoparasite species richness	0.06	0.08	35.76	0.82	0.42
Age	0.03	0.01	22.06	1.89	0.072
Neutrophil: lymphocyte ratio (NLR)					
Intercept	−1.07	0.16	30.02	−6.64	**<0.001**
Hemoparasite species richness	0.0003	0.07	32.66	0.004	0.997
Age	0.03	0.01	18.74	2.49	**0.022**

the corresponding blood smears, and species differentiation based on microfilaria morphology only is difficult, because the length ranges of both species may potentially overlap. This could have led us to misdiagnose infections in some cases and we might have missed statistically significant effects on Onchocercidae infections or hemoparasite species richness, as well as the effects of those on hematology values.

Sex did not influence infection with any single species, nor species richness, indicating that males are neither more susceptible to infections nor more exposed. Although sex-biased patterns of parasitism, including the parasite taxa examined in this study, seem to exist in many mammals (Klein, 2004), this effect does not seem to be universal (Kiffner et al., 2013). Specifically, sifakas do not display sexual size dimorphism (Kappeler, 1990), which has been proposed to relate to sex differences in parasite susceptibility in general (Moore and Wilson, 2002), while larger bodied hosts may also attract more vectors (Davies et al., 1991). Furthermore, male and female sifakas, like other lemurs with female dominance, exhibitit smaller differences in androgen levels than other mammals (von Engelhard et al., 2000; Drea, 2007), indicating that sex-specific endocrine–immune interactions may be less pronounced.

We tested whether host social group size influenced infection with each single hemoparasite species as well as hemoparasite species richness, but we did not find any significant effect. Group sizes in this study ranged from 2 to 7 individuals, and this range may have been too small to detect any differences, although small group-size differences should have a more pronounced effect on individual infection risk in small groups. Thus, our data do not support the encounter-dilution hypothesis, nor the opposing prediction that larger groups attract more vectors, which was supported by two studies of *Plasmodium* sp. prevalences across Neotropical primates with group sizes ranging from 3 to 29 individuals (Davies et al., 1991; Nunn and Heymann, 2005).

We also did not find any effect of seasonality, neither on *Plasmodium* sp. infections nor on infections with any of the other parasites, although fewer mosquitoes are present during the dry season. This indicates that infections are persistent for at least several months. Persistent infections as well as long developmental times of the parasite within the host, as might be the case for filarial nematodes, may mask seasonal effects.

Because of the opposite patterns of age-dependency of *Plasmodium* and *Babesia* infections, and because *Babesia* sp. infections were a significant negative predictor for *Plasmodium* sp. infections, we suggest that infection with *Babesia* sp. may be a natural protectant against *Plasmodium* sp. in this lemur species. As sifakas in Kirindy give birth during the dry season, when almost no mosquitoes are present, juveniles likely acquire *Babesia* sp. infections via tick-bites, probably of haemaphysaline ticks, which occur in western Madagascar (Rodriguez et al., 2012) and have been detected on sifakas during animal captures, before they become exposed to *Plasmodium* sp. Until babesiosis resolves, e.g. due to antibody-mediated immunity (Frolich et al., 2012), they seem to be naturally protected against *Plasmodium* sp. infections.

The suppression of malaria infections in the course of ongoing babesiosis has been investigated in rhesus macaques (*Macaca mulatta*) in laboratory settings (van Duivenvoorde et al., 2010), after it was found that a macaque infected with a *B. microti*-like parasite showed a suppressed *Plasmodium cynomolgi* infection (Wel et al., 2008). Heterologous immunity between the two parasites has also been observed in mice (Cox, 1978; Zivkovic et al., 1984). It has been proposed that the cross-protection is most likely due to immune responses such as the activation of a distinct class of pro-inflammatory monocytes, release of pro-inflammatory cytokines, and increased C-reactive protein levels, which potentially play an important protective role in malaria infections (Clark, 2001; Ansar et al., 2006; van Duivenvoorde et al., 2010). It has also been proposed that this heterologous immunity might be exploited for the development of malaria vaccines.

However, an interaction between *Plasmodium* and *Babesia* could also arise due to resource competition within the host. In fact, in humans it has been shown that co-infecting parasites mostly interact in this way (Griffiths et al., 2014). Both parasites invade and replicate in erythrocytes and both of them modify the erythrocytic membrane to achieve higher permeability, but do so using different mechanisms and at different time points after invasion (Alkhalil et al., 2007). Changes in membrane permeability induced by *Babesia* infection may thus make subsequent infection with *Plasmodium* sp. impossible and lead to a competitive advantage for *Babesia* sp. However, *B. microti* parasites suppressed *P. cynomolgi* parasitemia without altering the induction of initial anemia in rhesus macaques, suggesting the malaria parasites could enter erythrocytes during an infection with *Babesia* (van Duivenvoorde et al., 2010). The lack of positive microscopic observation of both *Babesia* and *Plasmodium* stages in the blood smears of infected individuals may partially result from technical limitations, but probably also indicates that parasitemia was generally low. This may suggest that if competition occurs, the parasites only compete for a narrow erythrocyte subset. While the precise mechanism of interaction remains unclear, the pattern found in our study provides the first indication of a possible protective effect of babesiosis on malaria infections in a wild population of primates naturally infected with both parasites, thus adding external validity to the above observation.

Finally, we did not find any signs of morbidity caused by the parasites tested in this study. Only animal age affected hematology values, a common pattern also in primates (McPherson, 2013). Packed cell volume was not significantly affected by infection with any single hemoparasite species nor by hemoparasite species richness, indicating that these parasites do not cause clinically relevant anemia. The absence of anemia may be explained by the long coevolutionary history between hemoparasites and their hosts, leading to a complete dependence of the parasite on the host for survival, which favours long-lasting asymptomatic infections with low parasitemia (Chauvin et al., 2009; Frolich et al., 2012). Furthermore, evidence has been found that co-infection with filarial nematodes may attenuate anemia associated with clinical malaria in humans (Dolo et al., 2012).

We did not detect signs of inflammation, either. Total plasma protein and the neutrophil-lymphocyte ratio were not affected in a statistically significant way. Acute inflammation usually causes an increase in plasma globulin concentrations, which may reflect in increased total plasma protein levels, as well as an increase in neutrophilic granulocytes (Thrall et al., 2006). However, the neutrophil-lymphocyte ratio may be affected in both directions by an excitement response with epinephrine release due to animal capture, as leucocytes are released from the marginal into the circulating pool (Thrall et al., 2006), which may have masked effects due to parasitism. Eosinophils typically show an increase as a response to helminth infection (Nutman, 2007), and high values have been measured in the course of filarial infections in dogs and humans (Mackenzie, 1980; Niwetpathomwat et al., 2007). Elevations may also occur in the course of babesiosis (Vercammen et al., 1997). The relative counts of eosinophils were not markedly elevated in any of the animals in this study, judging by comparison with previously reported values from both captive and wild *Propithecus* sp. populations (Bergeron and Buettner-Janusch, 1970; Junge and Louis, 2005; Irwin et al., 2010; Rasambainarivo et al., 2014). However, this conclusion remains preliminary as we were not able to obtain absolute leucocyte counts due to restrictions of fieldwork.

In conclusion, prevalences of hemoparasite infections in Verreaux's sifakas in Kirindy Forest, Madagascar, are significantly related to host age, probably shaped by interaction between *Babesia* sp. and *Plasmodium* sp., but independent of host sex, host group size and season. The possible cross-protection between *Babesia* sp. and *Plasmodium* sp. is a prime example of how biodiversity may affect the ecology of infectious diseases. Increasing evidence has accumulated that biodiversity may have a buffering role on disease transmission, while biodiversity loss is thought to increase transmission, disease incidence and disease emergence (Daszak et al., 2001; Ostfeld, 2009; Keesing et al., 2010; Vourc'h et al., 2012). In this case, the presence of tick-borne babesiosis seems to reduce *Plasmodium* sp. prevalence in a natural system, illustrating how within-host pathogen diversity may regulate the abundance of parasites. Infection status did not influence hematology values, suggesting that the parasites considered in this study do not cause clinically relevant anemia or inflammation, but that hosts and parasites are well-adapted to each other, resulting in persistent infections with low pathogenic potential.

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