Ecology of malaria infections in western lowland gorillas inhabiting Dzanga Sangha Protected Areas, Central African Republic

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(*Received 25 June 2014; revised 12 January 2015; accepted 16 January 2015; first published online 4 March 2015*)

SUMMARY

African great apes are susceptible to infections with several species of *Plasmodium*, including the predecessor of *Plasmodium falciparum*. Little is known about the ecology of these pathogens in gorillas. A total of 131 gorilla fecal samples were collected from Dzanga-Sangha Protected Areas to study the diversity and prevalence of *Plasmodium* species. The effects of sex and age as factors influencing levels of infection with *Plasmodium* in habituated gorilla groups were assessed. Ninety-five human blood samples from the same locality were also analysed to test for cross-transmission between humans and gorillas. According to a *cytB* PCR assay 32% of gorilla's fecal samples and 43·1% human individuals were infected with *Plasmodium* spp. All *Laverania* species, *Plasmodium vivax*, and for the first time *Plasmodium ovale* were identified from gorilla samples. *Plasmodium praefalciparum* was present only from habituated individuals and *P. falciparum* was detected from human samples. Although few *P. vivax* and *P. ovale* sequences were obtained from gorillas, the evidence for cross-species transmission between humans and gorillas requires more in depth analysis. No association was found between malaria infection and sex, however, younger individuals aged ≤6 years were more susceptible. Switching between two different *Plasmodium* spp. was observed in three individuals. Prolonged monitoring of *Plasmodium* infection during various seasons and recording behavioural data is necessary to draw a precise picture about the infection dynamics.

Key words: African great apes, malaria, lowland gorilla, *Plasmodium* spp.

INTRODUCTION

The range of non-human primates (NHPs), overlaps with areas where *Plasmodium* infection is endemic in humans. This has led to concerns that NHPs may represent a source of infection for humans (Prugnolle *et al*. 2011*a*; Rayner *et al*. 2011; Sundararaman *et al*. 2013). Until recently, only three *Plasmodium* species (*Plasmodium reichenowi*, *Plasmodium rodhaini* and *Plasmodium schwetzi*) were known to infect chimpanzees and gorillas (Blacklock and Adler, 1922; Adler, 1923), and among them *P. reichenowi* was the only one genetically characterized (Escalante and Ayala, 1994). However, newly introduced non-invasive fecal sampling combined with molecular diagnostics allows straightforward detection of circulating *Plasmodium* species and the study of their genetic diversity (Kaiser *et al*. 2010; Liu *et al*. 2010; Prugnolle *et al*. 2010). Latest studies have shown that a remarkably diverse array of *Plasmodium* species are widely distributed in NHPs in Africa (Duval and Ariey, 2012; Verhulst *et al*. 2012).

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doi:10.1017/S0031182015000086

Based on phylogenetic analysis of mitochondrial, apicoplast and nuclear genes of plasmodium species detected in gorillas and chimpanzees, six well defined host specific lineages are known to them. These lineages have been shown to belong in the subgenus *Laverania* (Liu *et al*. 2010). Among them, *Plasmodium adleri, Plasmodium praefalciparum*, and *Plasmodium blacklocki* have so far only been reported from gorillas. *Plasmodium praefalciparum* is of exceptional interest, as it is currently considered to be the direct predecessor of *P. falciparum,* the most pathogenic species in humans (Krief *et al*. 2010; Rayner *et al*. 2011; Verhulst *et al*. 2012; Sundararaman *et al*. 2013). Moreover, a single non-*Laverania* species, *Plasmodium vivax*, has also been reported from gorillas (Liu *et al*. 2010, 2014; Prugnolle *et al*. 2013; Sundararaman *et al*. 2013). Although an impressive amount of information about genetic diversity and distribution of *Plasmodium* species among NHPs has been obtained within the last few years, additional data on their biology and ecology and their interaction with their respective hosts is needed (Chapman *et al*. 2005; Prugnolle *et al*. 2011*a*). To date, only one study on chimpanzees has addressed the influence of a hosts ecology in determining their risk of infection with *Plasmodium* (De Nys *et al*. 2013). No such study has been conducted on the other species of NHP.

Western lowland gorillas have been habituated for the purpose of both research and eco-tourism (Blom *et al*. 2004; Doran-Sheehy *et al*. 2007). However, during the habituation process, individual behaviour (Klailova *et al*. 2010) and physiological wellbeing could be altered (Morton *et al*. 2013). Moreover, prolonged exposure of primates to humans may result in chronic stress, causing a reduction in immunity and increase the risk of pathogen transmission (Kalema-Zikusoka *et al*. 2005; Shutt *et al*. 2014). Field sites with habituated groups of NHPs offer a unique opportunity to study various aspects of their infectious diseases. Since 2007, as a part of the health monitoring efforts in Dzanga-Sangha Protected Areas (DSPA), we have been systemically surveying humans, gorillas and other wildlife for a broad spectrum of pathogens in order to assess their prevalence and cross-transmission (Sak *et al*. 2013; Hasegawa *et al*. 2014; Janatova *et al*. 2014; Shutt *et al*. 2014). DSPA is the first places to successfully habituate western lowland gorillas, this site is unique in its relatively dense gorilla population and of the number of groups under different stages of habituation. Two gorilla groups, named Makumba and Mayele, were added to the habituation programme in 2000 and 2005, respectively (Masi, 2009). Two other groups (Mata and Wonga) are under the process of habituation, and other unhabituated gorillas inhabit the same area. Data on the prevalence and genetic diversity of *Plasmodium* in gorillas at DSPA were discussed in the previous study (Liu *et al*. 2010), but without addressing the ecological aspects of this causative agent of malaria.

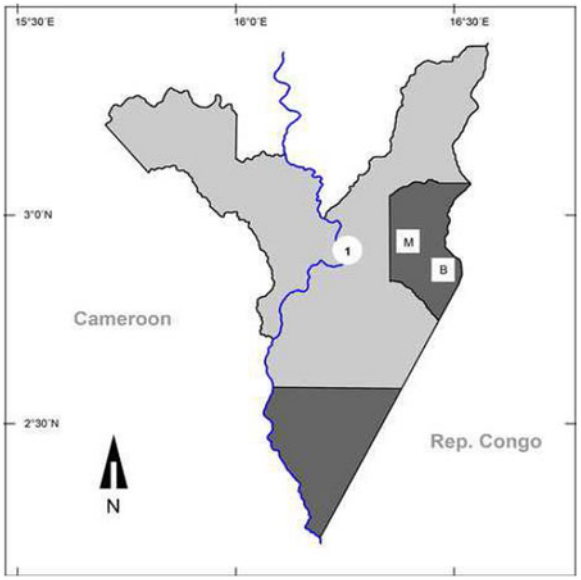

Fig. 1. Map of study site in Dzanga-Sangha Protected Areas, Central African Republic. Dzanga and Ndoki Sectors of the protected Dzanga Ndoki National Park (dark grey), Dzanga Sangha Dense Forest Special Reserve (light grey); location of villages Bayanga (1), Mossapoula and Yandumbé; research camp Mongambe (M); research camp Bai Hokou (B).

To answer some of these arising questions, we carried out a cross-sectional molecular survey to: (i) estimate the prevalence of *Plasmodium* among gorilla groups at different levels of habituation, (ii) to infer the genetic diversity of these *Plasmodium*, (iii) assess the possibilities of gorilla-human *Plasmodium* cross-transmission, and (iv) to investigate the effect of sex and age on the incidence of malarial infections in habituated lowland gorillas.

MATERIALS AND METHODS

Ethics statement

The research complied with the legal requirements of the Central African Republic (CAR) and adhered to the research protocol of DSPA. Collection of fecal samples from gorillas was non-invasive and did not cause any distress to the animals. The collection of human samples was approved by the Ethical Committee of the Biology Centre of the Czech Academy of Sciences (approval number: 1/2012). Import of samples into the EU was approved by the State Veterinary Authority of the Czech Republic.

Field site

This study was conducted in DSPA in the CAR (Fig. 1) and is co-managed by the CAR government

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and World Wildlife Fund (WWF). The complex is comprised of the strictly protected Dzanga-Ndoki National park (1222 km^2) and the multi-use Dzanga-Sangha Dense Forest Special Reserve (3159 km^2), where human activities are governed. Human population density in DSPA is low and estimated at around 6000 inhabitants (Remis and Jost Robinson, 2012). Estimated average rainfall is ~1400 mm year^{-1} with long rainy season intervals (March–May and August–November) and shorter dry seasons (Remis and Jost Robinson, 2012). In 1997, the primate habituation programme (PHP) was launched to habituate western lowland gorillas for tourism, at the same time providing researchers a unique opportunity to follow the gorillas on a daily basis and collect longitudinal sets of data from identified individuals within the habituated groups.

Sample collection

Sampling was carried out around the permanent PHP camps at Bai Hokou (33N 663109, 316187 UTM) and Mongambe (33N 654357, 322606 UTM). In total, 131 fecal samples were collected (Table 1), between August and October 2012. All fecal samples were immediately preserved in RNA*later*, stored in a freezer and subsequently shipped to Czech Republic, where they were kept at −20 °C until DNA extraction. The samples were collected from animals under different levels of habituation as shown below:

Habituated: Samples were collected from 9 and 13 individuals from two groups Makumba and Mayele, respectively. Both groups are regularly followed by two teams of BaAka trackers; approximately three trackers per team, Bantu assistants, as well as foreign researchers. Samples from identified individuals within habituated groups were collected on a monthly basis (16 individuals were sampled more than once), however, some samples were also collected from night nests and marked as unidentified in order to enable comparison of the occurrence of malaria infection in both under habituation and unhabituated groups.

Under-habituation. Two groups were also included in this study: the approximate number of individuals in the Mata group was eight and in the Wonga group there was 10–15, however we collected 28 and 11 fecal samples from Mata and Wonga group, respectively. Due to limited contact, samples were collected only from night nests. The Mata group was sampled three times, whereas the Wonga group was sampled only once. All the samples were marked as unidentified.

Unhabituated. The range of several unhabituated gorilla groups surrounded the research stations.

Table 1. Number of fecal samples collected and the identified *Plasmodium* spp.

Group	Status	No. of sample			Species identified				
		Identified	Unidentified	*n*/*N* (%)	*Plasmodium praefalciparum*	*Plasmodium adleri*	*Plasmodium blacklocki*	*Plasmodium vivax*	*Plasmodium ovale*
Makumba	Habituated	30	9	16/39 (41)	5	8	2	–	–
Mayele	Habituated	21	7	13/28 (46·4)	4	4	2	2	1
Wonga	Under-habituated	–	11	3/11 (27·3)	–	3	–	–	–
Mata	Under-habituated	–	28	3/28 (10·7)	–	2	–	1	–
Group 1	Unhabituated	–	6	3/6 (50)	–	2	1	–	–
Group 2	Unhabituated	–	3	2/3 (66·7)	–	1	1	–	–
Group 3	Unhabituated	–	2	0/2 (0)	–	–	–	–	–
Group 4	Unhabituated	–	14	2/14 (14·3)	–	1	1	–	–

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Table 2. Primer name, sequence and amplicons size

Primer	Sequence	PCR conditions			bp	Reference
DW2	5′-TAATGCCTAGACGTATTCCTGATTATCCAG-3′	95 °C	15′		~1220	Prugnolle
DW4	5′-TGTTTGCTTGGGAGCTGTAATCATAATGTG-3′	94 °C	30″			*et al*. (2010)
		60 °C	1′30″	×5		
		72 °C	1′30			
		72 °C	10′			
		10 °C	10′			
Cytb1	5′-CTCTATTAATTTAGTTAAAGCACA-3′	95 °C	5′		~558	Prugnolle
Cytb2B*	5′-GCTCTATCATACCCTAAAGG-3′	94 °C	30″			*et al*. (2010)
		44·9 °C	1′	×30		
		72 °C	1′			
		72 °C	5′			
		4 °C	~			
Cytb2	5′-ACAGAATAATCTCTAGCACC-3′	95 °C	5′		~516	Prugnolle
Cytb1A+	5′-CAAATGAGTTATTGGGGTGCAACT-3′	94 °C	30″			*et al*. (2010)
		58·9 °C	1′	×30		
		72 °C	1′			
		72 °C	5′			
		4 °C	~			

Cytb2B* primer designed in this study, amplifying first overlapping fragment.
Cytb1A+ primer designed in this study, amplifying second overlapping fragment.

We successfully sampled four of the unhabituated groups there, the group composition was unknown (samples collected: group 1, $n = 6$; group 2, $n = 3$; group 3, $n = 2$; group 4, $n = 9$). Sampling was carried out only once from night nests and all samples were marked as unidentified.

Blood samples. As part of a long-term health monitoring programme, 95 human blood samples were collected from residents of the local village of Bayanga, approximately 10 km outside of the National Park, including PHP trackers and assistants as well as park eco-guards; these samples were preserved in 96% ethanol and stored in a mobile freezer.

DNA extraction

Prior to DNA extraction, all fecal samples were processed in an equipped P3 laboratory, to remove RNA*later* as described elsewhere (Keele *et al*. 2006), and the extraction was performed with QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Total DNA was measured by Fluorometry, using a Qubit (Invitrogen Carlsbad CA). Genomic DNA from the human blood samples was extracted using the DNeasy blood & tissue kit (Qiagen) according to the manufacturer's instructions.

PCR and sequencing

For diagnostic purposes, a previously described nested PCR protocol was followed (Prugnolle *et al*. 2010). However, the second PCR reaction, designed to produce a ~930 bp fragment of the *Plasmodium* cytochrome *b* (*cytB*) gene was modified. A pair of internal primers amplifying two overlapping fragments (516 and 558 bp) of the same region of *cytB* was designed. Details of the primers used, fragment lengths and the modified PCR conditions are included in Table 2. For the first PCR, ca 2–50 ng of template DNA was used in a 25 μL reaction, containing 12·5 μL of PCR mix (Qiagen), 2·5 μL of solution Q (Qiagen) and 0·2 μL of each primer (DW2 and DW4) in 10 pmol concentration. For the second PCR, 3 μL of the first PCR product was used as a template in a 25 μL reaction mixture, also containing 12·5 μL common Master Mix (Top-Bio, Czech Republic) and 1 μL of each primer at the same concentration as in the first PCR reaction. The amplified products were subjected to 2% agarose gel electrophoresis in TAE buffer, excised, gel-purified using QIAquick gel extraction kit (Qiagen) and commercially sequenced in both directions by Macrogen. Human blood samples served as templates for the same *cytB* PCR reaction as that used for gorilla fecal samples.

Phylogenetic analyses

All sequences were manually checked using Chromas Pro 1.5 software (Technelysium, Ltd). Sequences were compared with previously published sequences using NCBI BLAST. Sequences were aligned using the ClustalW multiple alignment tool implemented in Bioedit Sequence Alignment Editor V.7.0.9.1. The best fitting model for maximum likelihood (ML) was general time reversible plus gamma distribution (GTR+Γ) for nucleotide as determined by jModelTest. A phylogenetic tree was constructed by ML and the corresponding bootstrap support values were obtained by PhyML software freely

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available at the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/).

Statistical analyses

To determine general *Plasmodium* prevalence among gorillas and humans, the rate of infection was calculated as a simple ratio, a 95% CI was estimated based on two methods as described previously (Newcombe, 1998). *Plasmodium* species prevalence in gorillas was calculated based on the proportion of positive fecal samples, while in humans the value was calculated based on the proportion of positive individuals. Gorilla sample data was analysed from two perspectives: (i) to compare malaria prevalence rate between groups that differ in levels of habituation, and (ii) the effect of sex and age on the probability of *Plasmodium* infection. For the effect of habituation and individual traits, we fitted two generalized linear mixed models (GLMM) with a binomial distribution. In the first model we used unidentified fecal samples collected from the night nests just once ($n = 16$ habituated, $n = 19$ under-habituation and $n = 25$ unhabituated) in order to compare the prevalence of *Plasmodium* species among gorillas according to their habituation status. Samples were categorized according to the groups (Makumba, Mayele, Mata, Wonga and unhabituated groups 1–4) and level of human-ape contact (habituated under-habituation and unhabituated). The random factor 'group' was nested into the fixed factor level of human-ape contact. In the second model; created in order to determine individual-trait factors that may underlay the incidence of malaria in gorillas, we included only identified samples from the two habituated groups collected during close follows of the animals (Makumba and Mayele) as sex and age of habituated animals are known. We treated group (fixed: Makumba, Mayele), sex (fixed: male, female), age (fixed, categories: infant/juvenile *vs* sub-adult/adult) and 'individual' (random effect). Age-classes were verified based on previously suggested categorization (Breuer *et al*. 2008). Due to the limited number of groups and the restricted number of individuals within each age class, age was pooled and categorized as young (infant/juvenile) *vs* old (sub-adult/adult). All statistical analyses were conducted in R (v. 2.13.1; R Development Core Team 2011).

RESULTS

Detection of Plasmodium DNA and species identification

In total, we examined 131 gorilla fecal samples and 95 human blood samples; *Plasmodium* spp. DNA was detected via PCR in 42 gorilla samples with an overall prevalence of 32%. According to the level of habituation, there were 4/16 (25%), 4/19 (21%) and 7/25 (28%) positive fecal samples from habituated, under-habituation and unhabituated groups, respectively. Habituation status had no effect on detection rate (GLMM: $z = 0{\cdot}277$, $P = 0{\cdot}871$). The results using *Cytochrome b* gene PCR-assay, also revealed high prevalence in human of 43·1% ($n = 41/95$).

We retrieved 41 unambiguous sequences out of 42 PCR amplicons. All *Laverania* species previously reported from lowland gorillas were detected from fecal samples in this study. *Plasmodium adleri* was most frequent ($n = 21$; 51%) followed by *P. praefalciparum* ($n = 9$; 21·9%) and *P. blacklocki* ($n = 7$; 17%). *Plasmodium praefalciparum* was present only in fecal samples collected from habituated individuals from both the Makumba and Mayele groups. In comparison, 38 sequences out of 41 PCR amplicons from human samples were unambiguously identified as *P. falciparum* with 99% similarity to KC175316 sequence from GenBank™. Sequences retrieved from both gorillas and humans were deposited in GenBank™ under these accession numbers (KM527127–KM527176).

Four samples containing *cytB* of non-*Laverania* species, namely *P. vivax* ($n = 3$; 7%) and *P. ovale* ($n = 1$; 2·4%) were also found. Both species were identified in the Mayele group, whereas only a single *P. vivax* case was found in the Mata group. One of the two samples containing *P. vivax* collected from the infant/juvenile Kaya (Mayele group) was shown to have 100% identity with KF618566 and AY791614 sequences obtained from human blood, as well as with KC140105 derived from *Anopheles vinckei*. The other sample was collected from an unidentified individual (Mata group) and shown to be identical with KF618562 from gorilla faeces (Cameroon) and KF591835 and JQ240429 from human blood. Due to poor quality of the third sequence we did not include it in further analysis. The single sample positive for *P. ovale* was collected from the infant/juvenile Ngobo (Mayele group) is characterized by 99% similar to HQ712053 from the human blood (tropical and subtropical regions), and GQ231520 and KC175307 from human blood (Cameroon).

The presence of this *P. ovale* sequence in *G. g. gorilla* was confirmed twice by using independent DNA extraction and PCRs. The dendogram (Fig. 2) shows the phylogenetic relationship of the newly obtained *cytB* sequence of *P. ovale* with relevant sequences of *P. ovale* from human and other *Plasmodium* species. Consistent topology was obtained by both maximum likelihood and Bayesian methods. The bootstrap value (96%) unequivocally support the placement of the *P. ovale* sequence into the *Plasmodium ovale wallikeri* clade, forming a monophyletic group together with sequences derived from humans, lemurs and chimpanzees.

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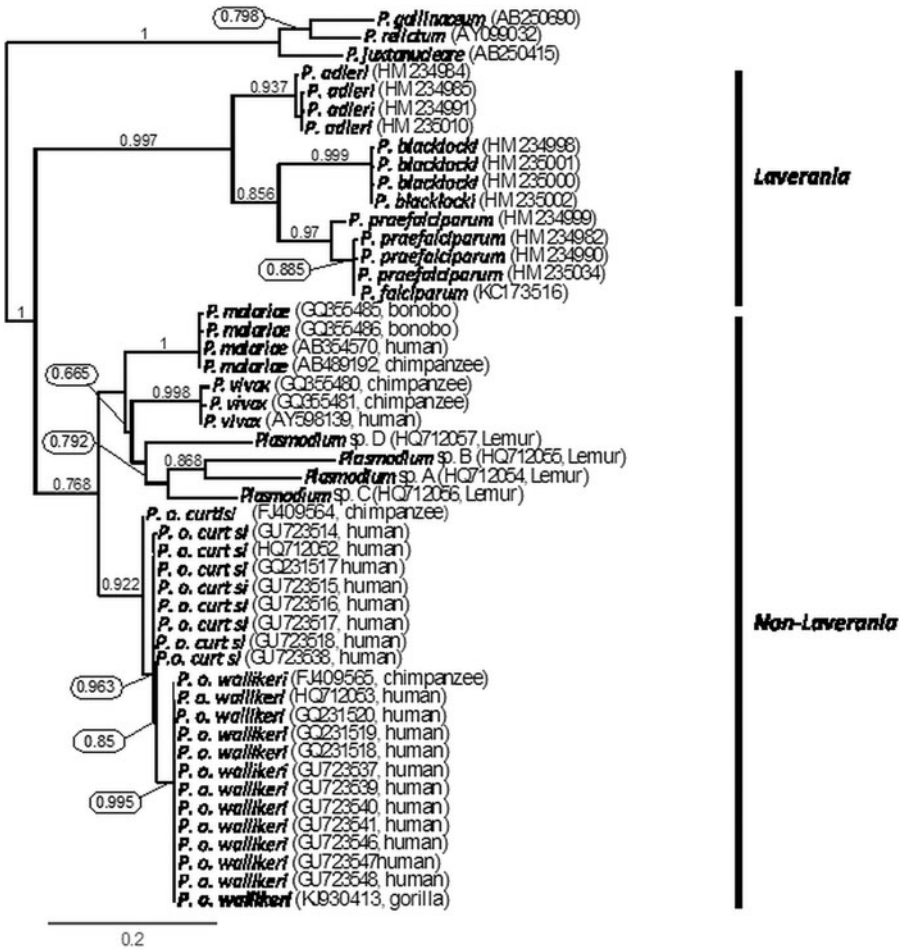

Fig. 2. Maximum likelihood (ML) phylogenetic tree of Plasmodium mitochondrial cytochrome b sequences (634bp). Numbers above branches denoted for bootstrap values with 1000 replicates. Newly identified sequences in this study are in bold.

Patterns of malaria infections in habituated gorillas

Fifty one fecal samples from 22 identified individuals from the habituated groups (Makumba, $n = 9$ and Mayele, $n = 13$) were collected and analysed (Table 3). *Plasmodium* DNA was detected in 25 samples (49%), of which 24 yielded a sequence that allowed determination of the *Plasmodium* species (Table 3). GLMM showed that age was the only significant factor influencing *Plasmodium* incidence (treatment contrasts given, infant/juvenile *vs* subadult/adult: $z = 1{\cdot}965$, $P = 0{\cdot}049$), while no effect of sex (treatment contrasts given: males *vs* females: $z = -0{\cdot}883$, $P = 0{\cdot}377$) or group (treatment contrasts given: Makumba *vs* Mayele: $z = 0{\cdot}315$, $P = 0{\cdot}753$) were observed. Throughout the sampling time, from all 22 habituated individuals, 11 gorillas were free of infection, whereas 11 individuals were positive at least once. Interestingly, fecal samples from individuals Ngobo and Liamba from the Mayele group changed in infection status over time. Additionally, infection with *P. adleri* following an infection with *P. praefalciparum* or vice versa was observed in samples collected from the individuals Tembo, Mobangi and Massoko from the Makumba group.

DISCUSSION

Early studies in the first half of the 20th century reported several malaria parasites in African NHPs (Blacklock and Adler, 1922; Adler, 1923; Rodhain, 1939; Garnham *et al*. 1956). Due to the highly endangered status of wild NHPs and ethical issues, further invasive studies are at present limited. Non-invasive approaches and the application of molecular methods, however, have dramatically improved our understanding of *Plasmodium* infections in terms of their prevalence and genetic diversity (Ollomo *et al*. 2009; Duval *et al*. 2010; Krief *et al*. 2010; Liu *et al*. 2010; Prugnolle *et al*. 2010). DSPA in the CAR is a key site for the habituation of western lowland gorillas and associated research, including studies of their (infectious) diseases (Cipolletta, 2003; Blom *et al*. 2004; Masi, 2009; Masi *et al*. 2009; Klailova *et al*. 2010; Sak *et al*. 2013; Hasegawa *et al*. 2014; Janatova *et al*. 2014; Shutt *et al*. 2014). Following the results from Liu

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Table 3. Patterns of *Plasmodium* spp. infection among individuals within habituated groups

					Sampling time				
Group	Individual	Age	Category	Sex	August	September	October		
	Mwangale	4	1	M	*Plasmodium adleri*				
	Elili	NA	2	F	NEG				
	Mapoki	NA	2	F		*Plasmodium praefalciparum*			
	Wuyia	1	1	M		NEG			
	Mopangu	10	2	M		NEG			
Mayele	Yoko	6	1	M	*Plasmodium blacklocki*	*Plasmodium blacklocki*			
	Sosa	8	2	M	*Plasmodium praefalciparum*	*Plasmodium praefalciparum*			
	Duma	NA	2	F	NEG	NEG			
	Mayele	29	2	M	NEG	NEG			
	Ngobo	6	1	M		NEG	*Plasmodium ovale*		
	Liamba	6	1	F		*P. adleri*	NEG		
	Lungu	2	1	NA		NEG	NEG		
	Kaya	2	1	M		*P. vivax*	*Plasmodium vivax*		
	Sopo	2	1	M	NEG				
	Bokata	6	1	F	*Plasmodium adleri*	*Plasmodium adleri*	*Plasmodium adleri*		
	Tembo	5	1	M	*Plasmodium adleri*	*Plasmodium adleri*	*Plasmodium praefalciparum*		
Makumba	Kunga	14	2	M	NEG	NEG	NEG		
	Mopambi	NA	2	F	NEG	NEG	NEG		
	Malui	NA	2	F	NEG	NEG	NEG	NEG	
	Massoko	8	2	F	*Plasmodium praefalciparum*	*Plasmodium praefalciparum*	*Plasmodium blacklocki*	*Plasmodium blacklocki*	
	Makumba	33	2	M	NEG	NEG	NEG	NEG	
	Mobangi	6	1	M	*Plasmodium adleri*	*Plasmodium adleri*	*Plasmodium adleri*	*Plasmodium praefalciparum*	NS

NEG, negative samples; NA, sex /age not available; NS, PCR positive but sequence was not obtained; 1, infant/juvenile; 2, sub-adult/adult.

et al. (2010), we conducted this study on the occurrence and diversity of malaria parasites among several groups of gorillas under different habituation levels, in order to investigate the impact of sex and age on infection. The *cytB* PCR assays identified a relatively high prevalence of *Plasmodium* spp. among the studied gorilla groups. As the detection of *Plasmodium* from fecal samples is less sensitive than from blood (Prugnolle *et al*. 2010), the actual prevalence would be expected to be higher. A 32% rate of infection in fecal samples reported herein is in agreement with previous finding from the same location (Liu *et al*. 2010). In contrast, gorillas from Cameroon and Gabon were found to have 21 and 11% prevalence, respectively (Duval *et al*. 2010; Prugnolle *et al*. 2010). Differences between the study sites could be attributed to small sample size bias, seasonality, sample group age composition, differential exposure to the appropriate vectors and other ecological factors (Verhulst *et al*. 2012).

Lowland gorillas are known to host *P. praefalciparum, P. adleri* and *P. blacklocki*, as well as a *P. vivax*-like isolate that belongs to the non-*Laverania* species (Liu *et al*. 2010; Prugnolle *et al*. 2013). All *Laverania* species known from gorillas were detected in our study site, with *P. adleri* being the most prevalent. The relatively high frequency of *P. adleri* may be attributed to mosquito host preference (Rayner *et al*. 2011; Verhulst *et al*. 2012), and also to varying exposure to different *Anopheles* mosquitoes transmitting *Plasmodium* species (Bray, 1958; Collins, 2012). The switching of infections observed among samples from three habituated individuals from Makumba group indicates that gorillas are infected with one or more *Plasmodium* species simultaneously and PCR will detect the species with the highest level of parasitaemia (Mueller *et al*. 2007). Interestingly, partial *cytB* sequences from three samples obtained from habituated and under-habituation groups were identical to *P. vivax* or 99% similar to *P. ovale*. Previously, a *P. vivax*-like sequence has been reported from gorillas at DSPA (Liu *et al*. 2010), however, to our knowledge, this is the first molecular evidence of *P. ovale* sequence from a gorilla. Since our *P. vivax* and *P. ovale* sequences were retrieved from gorilla groups that have close contact with humans, additional sequence information is needed to clarify the exact phylogenetic relationship with corresponding human *Plasmodium* species (Prugnolle *et al*. 2011*a*; Rayner *et al*. 2011; Verhulst *et al*. 2012).

Indeed, applying the phylogenetic concept of species has a potential to define cryptic taxa in morphologically indistinguishable organisms, either in situations when morphological data are unavailable or hardly obtainable, as in the case of *Plasmodium* spp. from NHPs. Currently there are two non-recombinant forms of *P. ovale,* described using the phylogenomics: *P. o. curtisi* (classical type) and *P. o. wallikeri* (variant type) (for more details about proposed taxonomy see (Win *et al*. 2004; Sutherland *et al*. 2010). These two forms have been identified from humans (Win *et al*. 2004; Sutherland *et al*. 2010) and chimpanzees (Duval *et al*. 2009), however, they have not yet been recorded from gorillas. So far, most of the described diversity of *Plasmodium* in NHPs refers to subgenus *Laverania,* showing several strictly host specific species/genotypes, closely related to *P. falciparum* (Liu *et al*. 2010; Pacheco *et al*. 2013; Sundararaman *et al*. 2013).

On the contrary, only limited diversity was described within the subgenus *Plasmodium* (often referred to as non-*Laverania* species), including *P. vivax* and *P. ovale* in gorilla and chimpanzee (Liu *et al*. 2010; Prugnolle *et al*. 2013) and possibly an accidental finding of *Plasmodium malariae* in a chimpanzee imported to Japan (Hayakawa *et al*. 2009). The *P. ovale* sequence obtained in our study nested within the *P. o. wallikeri* clade together with GenBank sequences from human and chimpanzee. This might indicate that humans are the source of infection of both gorillas and chimpanzee. However, it is also possible, that thorough investigation of diversity of non-*Laverania* species would reveal the existence of host specific lineages, similar to *Laverania*.

Close contact between humans and wildlife may facilitate transmission of a range of pathogens, ranging from respiratory viruses (Köndgen *et al*. 2008), to protists *Giardia intestinalis* (Sak *et al*. 2013) and helminths *Necator americanus* (Hasegawa *et al*. 2014) and malaria should not be an exception (Baird, 2009; Prugnolle *et al*. 2011*b*). Furthermore, the recent finding of chloroquine resistance *P. falciparum* strain in chimpanzees, suggest that NHPs may act as a natural reservoir of human malaria (Pacheco *et al*. 2013). The increased level of NHP-human contact, over the course of habituation likely increases chronic stress, compromising immune function and making individuals more vulnerable to infectious diseases (Kalema-Zikusoka *et al*. 2005; Rothman *et al*. 2008; Shutt *et al*. 2014). However, although it was recently shown that habituated and individuals undergoing habituation have higher levels of fecal glucocorticoid metabolites (implying increased level of stress hormones (Shutt *et al*. 2014)), we did not find any effect of habituation on the incidence of malaria infections.

Parasite distribution may be affected by a range of host characteristics such as age, sex and peculiarities of parasite life cycles (Schmid-Hempel and Koella, 1994; Müller-Graf *et al*. 1996). The habituation of the Makumba and Mayele groups began in 2000 and 2005, respectively. Empirical data (age, sex etc.) on the identified group members provides a unique opportunity to evaluate the effect of these

https://doi.org/10.1017/S0031182015000086 Published online by Cambridge University Press

epidemiological drivers on malaria infections. Previous studies of human malaria have provided some contradictory results in this respect, as some have reported a positive association between males and infection (Wildling *et al*. 1995; Pathak *et al*. 2012), while others have shown females to be more infected (Goselle *et al*. 2009). Consistent with the recent findings from wild chimpanzees (De Nys *et al*. 2013), we revealed no effect of host sex on the occurrence of malaria infection.

In humans, the pattern of malaria infection differs by age (Doolan *et al*. 2009; De Beaudrap *et al*. 2011). The only study that addressed the effect of age on malaria infection in wild chimpanzees showed that young individuals are more susceptible (De Nys *et al*. 2013), which agrees with the results from studies on humans (De Beaudrap *et al*. 2011) and semi-captive orangutans (Reid *et al*. 2006). In agreement with these findings, we also found young gorillas were more likely to be infected with *Plasmodium* spp. than older individuals. However, such higher prevalence in young animals may also be explained by a higher parasitaemia leading to an increased chance of fecal detection.

The potential risk of cross-transmission of *Plasmodium* infections between humans and NHPs has been highlighted in previous studies (Duval *et al*. 2010; Liu *et al*. 2010; Prugnolle *et al*. 2010, 2011*b*; Sundararaman *et al*. 2013), although such a transmission has never been proven. Since the data from gorillas and chimpanzees is limited, more elaborate longitudinal studies joining the diversity of *Plasmodium* spp. in humans and great apes living in sympatry together with vector feeding ecology is mandatory to sort out the role of humans/NHPs as a reservoir of *P. ovale* (Baird, 2009; Rayner *et al*. 2011; Duval and Ariey, 2012; Krief *et al*. 2012; Pacheco *et al*. 2013). In our study, none of the sequences obtained from gorillas were identified as *P. falciparum* and similarly we did not identify any primate *Laverania* species from humans. The detection in four samples of partial *cytB* sequences identical to *P. vivax* and *P. ovale* calls for further exploration.

Our inability to quantify the degree of infection, our poor understanding of the origin and relevance of *Plasmodium* DNA in faeces, together with a high risk of false negativity confounds stronger conclusions. Similarly, the assessment of the fine nuances in the health status of free ranging gorillas and/or chimpanzees makes evaluation of the real impact of malaria on infected individuals rather elusive. Still, long term monitoring of habituated individuals, systematic recording of behavioural health data, including non-invasive body temperature measurement (Jensen *et al*. 2009) and identification of mosquito vectors in their natural habitat are factors of significance that will undoubtedly improve our understanding of the ecology of their malaria.

ACKNOWLEDGEMENTS

The authors are grateful to the Ministre de l'Education Nationale, de l'Alphabetisation, de l'Enseignement, Superieur et de la Recherche of the Central African Republic for providing the research permits required to conduct this research; World Wildlife Fund and DSPA administration for granting research approval and assistance with obtaining permits; and the Primate Habituation Programme for providing logistical support in the field. The authors would like to thank the staff of DSPA, assistants and BaAka trackers, and Petr Lany for access to the P3 laboratory. The authors greatly appreciated help from Erhan Yalcindag and Sebastian Calvignac-Spencer during optimization of our PCR protocol. We would like to thank Peter Stuart for proofreading the manuscript.

FINANCIAL SUPPORT

This work was funded by IGA 1230-21-IG 121 231, grant of the Czech Academy of Sciences M200961204 and the EurNegVec COST Action TD1303. This project was further co-financed from European Social Fund and state budget of the Czech Republic (project OPVK CZ.1.07/2.3.00/20.0300). M.A.Q., was supported by project OPVK CZ.1.07/2.3.00/30.0014. J.L., who is a fellow of the Canadian Institute for Advanced Research, was supported by the Praemium Academiae award.

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https://doi.org/10.1017/S0031182015000086 Published online by Cambridge University Press

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