Non-invasive molecular epidemiology of malaria infection in wild chimpanzees

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LIST OF ABBREVIATIONS

AGA	African great apes
An.	Anopheles
BLAST	Basic Local Alignment Search Tool
Bp	base pair
C1	chimpanzee *Laverania* clade C1,
C2	chimpanzee *Laverania* clade C2
C3	chimpanzee *Laverania* clade C3
CD40	Cluster of differentiation 40
CI	confidence interval
cox 1	cytochrome c oxidase subunit I
cyt b	cytochrome b
d.f.	degree of freedom
CR	mitochondrial control region
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
EMBL	European Molecular Biology laboratory
F	female
G1	gorilla *Laverania* clade G1
G2	gorilla *Laverania* clade G2
G3	gorilla *Laverania* clade G3
GLMM	Generalized Linear Mixed Model
G. g.	*Gorilla gorilla*
G. b.	*Gorilla beringei*
G6PD	glucose-6-phospate dehydrogenase
Ha. sp.	*Haemoproteus* species
ID	individual identity
L. sp.	*Leucocytozoon* species
M	male

min	minute
µL	microliter
mM	millimolar
µM	micromolar
mtDNA	mitochondrial DNA
MPI	Max Planck Institute for Evolutionary Anthropology
NHP	non-human primate
P. t.	*Pan troglodytes*
Pa. sp.	*Parahaemoproteus* species
P. spp.	*Plasmodium* species
PCR	polymerase chain reaction
qPCR	quantitative PCR
RKI	Robert Koch Institute
RNA	ribonucleic acid
rRNA	ribosomal RNA
s	second
s.e.	standard error
SGA	single genome amplification
SIV	simian immunodeficiency virus
SIVcpz	simian immunodeficiency viruses infecting chimpanzees
TCP	Taï Chimpanzee Project
TNP	Taï National Park
UNG	uracil N glycosylase
var	variant surface antigen

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GENERAL INTRODUCTION

African great apes (AGA) have been known to be natural hosts of malaria parasites since the early 1920's when researchers first described various *Plasmodium* species (*P.* spp.) infecting chimpanzees (*Pan troglodytes*) and gorillas (*Gorilla* sp.), which are closely related to those infecting humans (Reichenow, 1920).

To this day it remains difficult to date the actual origins of the malaria parasites which infect humans (Sharp *et al.*, 2013), as well as their emergence in human populations. However, the expansion of *P. falciparum* for instance, the most virulent *Plasmodium* parasite (White, 2003), is believed to have started in Africa at least thousands or even tens of thousands years ago (Carter and Mendis, 2002; Rich *et al.*, 2009; Tanabe *et al.*, 2010; Volkman *et al.*, 2001), and to have played a significant role on recent human evolution (Carter and Mendis, 2002). Malaria is considered a major public health concern; it is currently geographically widespread and constitutes a very heavy burden for human health (WHO, 2015). Malaria in great apes, our close evolutionary relatives, is thus an important topic of investigation, with the hope to better understand human malaria, but also the implications of malaria for great ape populations' health.

Research was initially limited to the study of captive or even experimental AGA (Bray, 1960; Contacos *et al.*, 1970; Garnham *et al.*, 1956; Rodhain and Dellaert, 1943; Rodhain, 1940, 1936; Schwetz, 1934), but the development of molecular diagnostic tools and, later on, the ability to use non-invasive samples (i.e. the discovery that malaria parasite genetic material can be found in faecal samples of primates (Prugnolle *et al.*, 2010), opened opportunities for further research in wild ape populations, allowing for more accurate identifications, discovery of additional species in chimpanzees and gorillas and the study of their phylogenetic relationships (Kaiser *et al.*, 2010; Liu *et al.*, 2014, 2010). This recently provided evidence that the human *P. falciparum* has a gorilla origin (Liu *et al.*, 2010), and that *P. vivax* originally emerged in Africa (Liu *et al.*, 2014).

Despite all the efforts made so far to describe the malaria parasite diversity, host specificity and evolutionary history, there is still very little knowledge on the epidemiology, ecology and biology of great ape malaria. The pathogenicity of malaria parasite infection in the wild is for instance totally unknown.

The present study was undertaken in order to shed light onto some of these aspects, starting with basic epidemiological characteristics such as the determinants of malaria

infection. Based on information from human malaria (Brabin, 1983; Doolan *et al.*, 2009; Whitworth *et al.*, 2000; WHO, 2015), the first logic question to arise is whether children, pregnant females and immunocompromised individuals also constitute the most vulnerable groups to malaria infection in wild chimpanzees, our genetically closest relatives. The availability of non-invasive diagnostic methods but also the existence and accessibility to habituated AGA communities to collect the necessary data made it conceivable to try and answer these questions in apes living in natural conditions. This work was thus initiated with the main objective to contribute to the knowledge on AGA malaria by looking at the effects of age and pregnancy on susceptibility to malaria parasite infection, but also to test the feasibility of non-invasively answering such "fine-scale" type of question and to provide methodological experience, which can eventually be applied to the study of other microorganisms.

This study was carried out in collaboration with the Max Planck Institute for Evolutionary Anthropology (MPI), Leipzig, for the field work, and the Robert Koch Institute (RKI), Berlin, for the laboratory work. Samples and data stemmed from one year of fieldwork on a habituated western chimpanzee (*P. t. verus*) community (Taï Chimpanzee Project (TCP), Taï National Park (TNP), Côte d'Ivoire, MPI), as well as from a large collection taken from the same community during the previous 12 years in the frame of a long-term health project instigated by Dr. Leendertz (RKI) in collaboration with MPI (Fabian H Leendertz *et al.*, 2006).

It led to the publication of two scientific articles on the effects of age and pregnancy on malaria infection in wild chimpanzees, as well as a methodological paper. This study follows two preceding malaria studies which initially confirmed the prevalence of malaria parasite infection in the same study population of chimpanzees (Kaiser *et al.*, 2010; Rich *et al.*, 2009), and falls within a more comprehensive and ongoing investigation on AGA malaria epidemiology, ecology and biology. On a broader scale, it contributes to the exploration of how infectious diseases have and still do influence evolution of species belonging to the Hominidae family.

After an overview of the existing literature in order to identify research questions and state the objectives, the research outcomes are exposed under the form of three published scientific papers, and general conclusions are finally discussed.

CHAPTER 1

STATE OF THE ART

1.1 Malaria parasite infection in wild chimpanzees and other wild African great apes

1.1.1 Parasite diversity, distribution and prevalence

Five *Plasmodium* species are classically known to infect humans and cause malaria: *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae* (White, 2003), historically considered "human" parasites, and *P. knowlesi* which is of zoonotic origin (Lee *et al.*, 2011). *P. falciparum* is widespread, with infections occurring in a large number of South American, African, Central and South East Asian countries. It is the most common malaria parasite in sub-Saharan Africa and is responsible for most of the severe malaria cases (White, 2003). *P. vivax* also has a wide geographical distribution, but a very low prevalence in Western and Central Africa due to the high proportion of the Duffy negative phenotype in the human population, which confers resistance against this parasite (Culleton *et al.*, 2008). *P. malariae* distribution generally matches the one of *P. falciparum* (Collins and Jeffery, 2007) and *P. ovale* is mainly present in sub-Saharan Africa and western Pacific islands (Collins and Jeffery, 2005). *P. knowlesi* occurs in Southeast Asia where wild macaques act as natural reservoir (Lee *et al.*, 2011).

Malaria parasites naturally infecting AGA were initially described in the 1920's by Reichenow (Reichenow, 1920) and then studied further and experimented on by various researchers such as Blacklock and Adler (Adler, 1923; Blacklock and Adler, 1922), Schwetz (Schwetz, 1934), Rodhain (Rodhain and Dellaert, 1943; Rodhain, 1940, 1936), Brumpt (Brumpt, 1939), Garnham (Garnham *et al.*, 1956), Bray (Bray, 1960, 1958a), and Contacos (Contacos *et al.*, 1970). Based on morphological features, three distinct *Plasmodium* species, considered similar to the human parasites, were identified in west and central Africa: *P. falciparum*-like and *P. ovale* or *vivax-like parasites* in both chimpanzees and gorillas, and *P. malariae*-like parasites in chimpanzees (Reichenow, 1920). These were subsequently named *P. reichenowi* (Blacklock and Adler, 1922), *P. schwetzi* (Brumpt, 1939) and *P. rodhaini* (Brumpt, 1939), respectively. *P. falciparum* and *P. reichenowi*, considered relatively distinct from other known *Plasmodium* species, were grouped separately under the *Laverania* subgenus (Bray, 1958b).

From 2009 onwards, with the increased use of molecular diagnostic tools (deoxyribonucleic acid (DNA) amplification and genome sequencing) and eventually non-invasive samples, studies on malaria parasites infecting great apes flourished, leading to the description of a higher diversity of parasites than previously thought, and giving clearer insight into the possible origins and evolutionary history of malaria infection in humans. To date, at least six *Plasmodium* species have been shown to be endemic in wild living chimpanzees: *P. reichenowi*, *P. gaboni*, *P. billcollinsi* and *P. ovale*, *vivax* and *malariae*-like parasites (putative nomenclature proposed by Rayner *et al.* (2011) and used in the present work). Western lowland gorillas (*Gorilla gorilla gorilla*) were are also found to naturally host five parasites species (*P. praefalciparum, P. adleri*, *P. blacklocki* and *P. vivax* and *ovale*-like parasites), while so far natural infections of bonobos (*Pan paniscus*) have not been found (**Figure 1.1 and 1.2**). Together with 3 clades of gorilla *Plasmodium* parasites, which also include the human *P. falciparum*, the chimpanzee *P. reichenowi*, *P. gaboni*, and *P. billcollinsi*, are phylogenetically grouped into the *Laverania* subgenus (Liu *et al.*, 2010; Rayner *et al.*, 2011).

1.1.1.1 Laverania parasites

Due to its morphological relatedness with *P. falciparum*, the most virulent malaria agent in humans, *P. reichenowi* was the first great ape *Plasmodium* species to be molecularly characterized. It was isolated and studied from a captive chimpanzee in the USA (Centers for Disease Control) (Collins *et al.*, 1986) and subsequently used in several genetic studies (Escalante and Ayala, 1994; Jeffares *et al.*, 2007; Lal and Goldman, 1991; Okenu *et al.*, 2000; Rayner *et al.*, 2004). Its presence in wild chimpanzee populations has since then been confirmed for all four chimpanzee subspecies: western chimpanzees (*P. t. verus*) (Kaiser *et al.*, 2010; Rich *et al.*, 2009), Nigeria-Cameroon chimpanzees (*P. t. ellioti*) (Liu *et al.*, 2010; Prugnolle *et al.*, 2010), central chimpanzees (*P. t. troglodytes*) (Boundenga *et al.*, 2015; Liu *et al.*, 2010; Prugnolle *et al.*, 2010) and eastern chimpanzees (*P. t. schweinfurthii*) (Kaiser *et al.*, 2010; Krief *et al.*, 2010; Liu *et al.*, 2010).

Until the discovery of other *Laverania* parasites, *P. reichenowi* was known as the closest relative of *P. falciparum* (Escalante and Ayala, 1994; Escalante *et al.*, 1995). A number of phylogenetic analyses have led to various hypotheses on the origin of the human *P.*

falciparum: 1- the co-speciation hypothesis, according to which the two *Plasmodium* species diverged from a common ancestor at the same time as the chimpanzee and human lineages diverged, about 6 to 10 million years ago (Escalante and Ayala, 1994; Escalante *et al.*, 1995; Hayakawa *et al.*, 2008; Hughes and Verra, 2010) and 2- the hypothesis of a more recent chimpanzee origin through a single host transfer of *P. reichenowi* from chimpanzee to human. The latter theory was supported by the low genetic polymorphism of *P. falciparum* fully included into the broader genetic diversity of malaria parasite lineages, which the authors collectively considered as forming the species *P. reichenowi* (Prugnolle *et al.*, 2010; Rich *et al.*, 2009).

Over the past six years, other great ape *Plasmodium* species from the *Laverania* subgenus have been discovered and characterized. *P. gaboni*, a relative of *P. falciparum* and *P. reichenowi*, was first described by Ollomo *et al.* (2009) in two pet chimpanzees (*P. t. troglodytes*) in Gabon and its presence was then confirmed in wild populations of *P. t. verus* (Kaiser *et al.*, 2010; Rich *et al.*, 2009), *P. t. troglodytes* (Boundenga *et al.*, 2015; Liu *et al.*, 2010; Prugnolle *et al.*, 2010), *P. t. ellioti* (Liu *et al.*, 2010) and *P. t. schweinfurthii* (Kaiser *et al.*, 2010; Liu *et al.*, 2010). A similar parasite was described by Krief *et al.* (2010) in two wild chimpanzees (*P. t. schweinfurthii*), and referred to as *P. billbrayi* but, according to Rayner *et al.* (2011), not sufficiently divergent from *P. gaboni* to term it as a separate species. They also detected another *Laverania* parasite in *P. t. schweinfurthii*, referred to as *P. billcollinsi*, since then also confirmed to occur in wild *P. t. troglodytes* (Boundenga *et al.*, 2015; Liu *et al.*, 2010) and *P. t. ellioti* (Liu *et al.*, 2010).

When several studies started using non-invasive biological material (faeces) to study malaria in wild AGA and successfully recovered malaria parasite DNA (Kaiser *et al.*, 2010; Liu *et al.*, 2010; Prugnolle *et al.*, 2010), investigations in wild populations expanded and led to the further discovery of new great ape *Plasmodium* species as well as substantial indication that *P. falciparum* originated from gorillas rather than from chimpanzees (Liu *et al.*, 2010). Initially, Prugnolle *et al.* (2010) found two new lineages in wild western lowland gorilla (*G. g. gorilla*), one closely related to *P. reichenowi* and *P. falciparum*, the other to *P. gaboni*. They also found sequences believed to be *P. falciparum* in two samples of wild western gorillas (*G.g. dielhi* and *G.g. gorilla*), which is the first time such sequences were reported in non-human primates (NHP). Finally, a broad-scale epidemiological study using a large collection

of faecal samples (nearly 3,000) gathered from wild chimpanzees (*P. t. ellioti*, *P. t. troglodytes* and *P. t. schweinfurthii*), bonobos (*Pan paniscus*), and eastern (*G. beringei graueri*) and western (*G. g. gorilla*) lowland gorillas across central Africa, and testing for a greater set of genomic regions, clarified the picture of the diversity of *Laverania* parasites circulating in wild AGA (Liu *et al.*, 2010). Six major clades of such parasites, formed by three species infecting chimpanzees (*P. reichenowi*, *P. gaboni*, *P*. *billcollinsi*) and three species infecting western lowland gorillas (*P. adleri* and *P. blacklocki*, *P. praefalciparum*, nomenclature proposed by Rayner *et al.*, (2011)), were confirmed or newly identified (**Figure 1.2**). No malaria parasites were detected in eastern lowland gorillas (*G. b. graueri*) and bonobos (*Pan paniscus*). The description of *Laverania* diversity in wild AGA further showed that, contrary to previous expectations, the entire diversity of human-derived *P. falciparum* sequences was encompassed by one of the clades formed by malaria parasites from western gorillas (*P. praefalciparum*), not from chimpanzees. This striking result is currently considered evidence of an ancestral zoonotic event, whereby the gorilla malaria parasite *P. praefalciparum* would have given rise to *P. falciparum* in humans (Liu *et al.*, 2010; Sundararaman *et al.*, 2013).

P. praefalciparum was also found in one pet monkey (*Cercopithecus nictitans*) (Prugnolle *et al.*, 2011b), but a subsequent large-scale survey failed to detect any *Laverania* parasite infection in this species in the wild (Ayouba *et al.*, 2012).

P. falciparum infection has been reported in captive chimpanzees (Duval *et al.*, 2010) and bonobos (Krief *et al.*, 2010). However, the absence of *P. falciparum* sequences in a large number of wild chimpanzee and bonobo samples (Liu *et al.*, 2010) strongly supports the idea that these infections resulted from cross-transmission of *P. falciparum* from humans during captivity (Rayner *et al.*, 2011). The two *P. falciparum* sequences that were found in wild gorilla samples by Prugnolle *et al.* (2010) are too short to distinguish between the human and gorilla parasites.

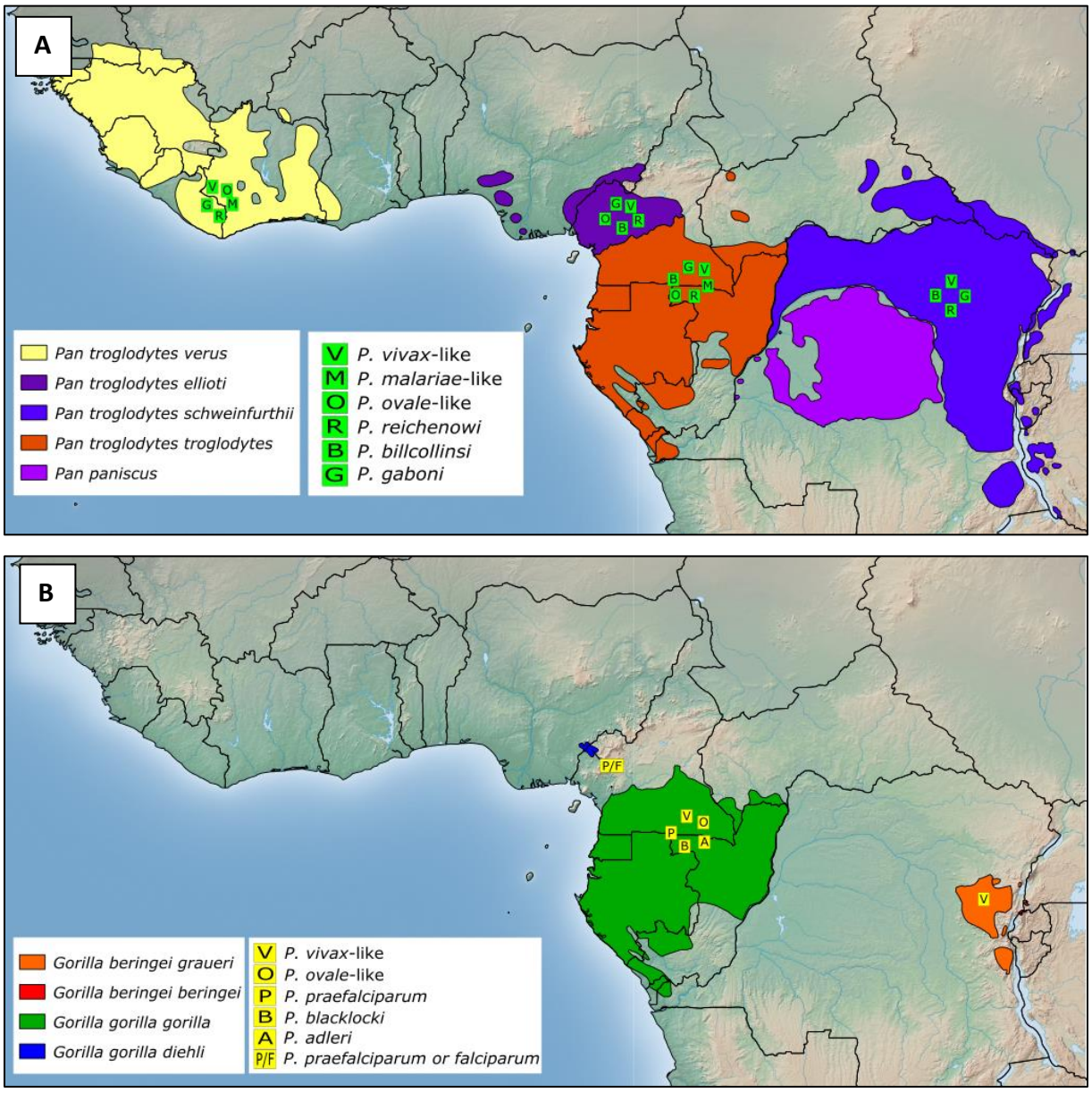

Figure 1.1. Distribution of *Plasmodium* species detected in wild chimpanzees and gorillas

Plasmodium species symbols placed on the geographical range of different chimpanzee subspecies (A) and gorilla species/subspecies (B) indicate that the assigned *Plasmodium* species was, to this date, detected in at least one sample of the relevant great ape species/subspecies. Positions of the *Plasmodium* symbols do not represent specific field site locations.

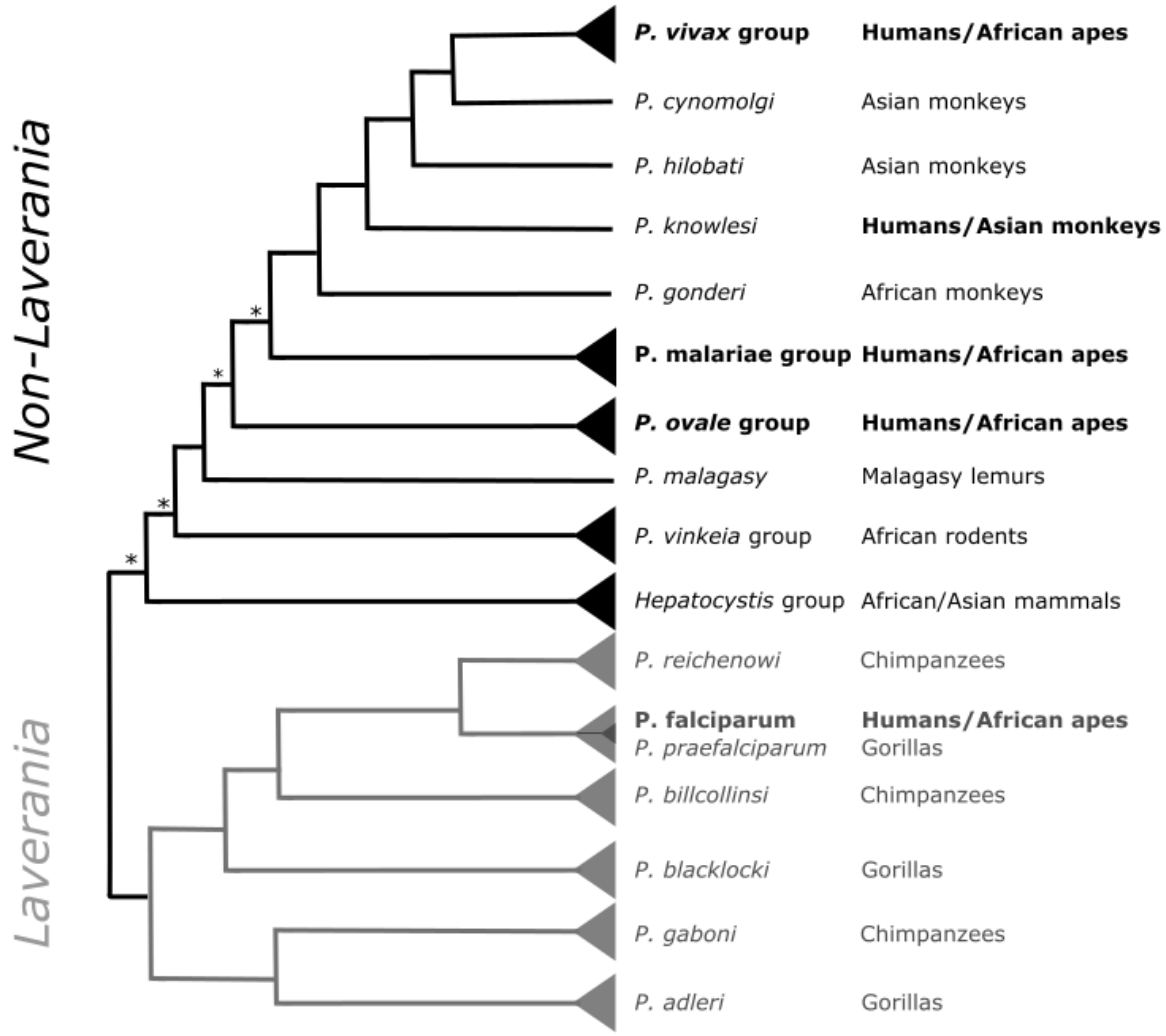

Figure 1.2. Schematic phylogeny of mammal *Plasmodium* and *Hepatocystis* parasites (based on Figure 1 from Duval *et al.* 2012). Parasites infecting humans are in bold. Grey colors represent *Laverania* parasites and black color non-*Laverania* parasites. *Unresolved nodes.

1.1.1.2 Non-Laverania parasites

Molecular studies confirmed that AGA also naturally harbour non-*Laverania Plasmodium* species related to the human *P. vivax*, *P. ovale* and *P. malariae* parasites (Kaiser *et al.*, 2010; Liu *et al.*, 2014, 2010; Mapua *et al.* 2015), initially thought to be human specific (**Figure 1.1 and 1.2**). Parasites genetically close to the human non-*Laverania* strains also circulate in

various Asian (Escalante *et al.*, 2005) and New World (southern Mexico, Central and South America) (Tazi and Ayala, 2011) monkey species. The phylogenetic relationships between all these parasites as well as their evolutionary origins are not fully elucidated yet.

Due to the public health importance and intriguing geographical pattern of *P. vivax* infection in humans, *P. vivax* infection in AGA has been drawing more attention compared to the two other non-*Laverania* lineages. *P. vivax* is widespread in Asia and Latin America, where it constitutes the main cause of malaria in humans (Mendis *et al.*, 2001), but has a very low prevalence in western and central Africa due to the high proportion of the Duffy negative phenotype in the human population, which confers resistance against this parasite through the absence of chemokine receptors on the erythrocytes (Culleton *et al.*, 2008). It is, however, sometimes described in travellers who have visited these regions (Broderick *et al.*, 2015; Gautret *et al.*, 2001; Mühlberger *et al.*, 2004; Skarbinski *et al.*, 2006).

Phylogenetic analyses have initially suggested that *P. vivax* originated in Asia from an ancestral macaque *Plasmodium* species (Escalante *et al.*, 2005; Mu *et al.*, 2005). *P. simium*, a New World monkey parasite, has also been proposed as a potential ancestor of *P. vivax*, given its high genetic similarity which is suggestive of a recent host transfer between New World monkeys and humans, although the direction of this cross-species transmission remains uncertain (Escalante *et al.*, 2005; Tazi and Ayala, 2011). Finally, the near fixation of the Duffy negative phenotype in sub-Saharan human populations has been supporting the hypothesis of an African origin of this parasite with positive selection of the Duffy negativity through long time exposure (Carter, 2003). Recently, several studies identified *P. vivax*-like parasites in wild AGA (*P. t. schweinfurthii*, *P. t. verus*, *G. g. gorilla*) (Kaiser *et al.*, 2010; Liu *et al.*, 2010), and phylogenetic analysis of strains found in captive AGA indicated that these were a genetically distinct clade from the parasites circulating in humans (Prugnolle *et al.*, 2013). A subsequent broad non-invasive epidemiological study in central Africa confirmed the endemicity of *P. vivax*-like parasites in chimpanzees (*P. t. troglodytes*, *P. t. ellioti and P. t. schweinfurthii*) and western (*G. g. gorilla*) and eastern (*G. b. graueri*) lowland gorillas (no malaria parasites were detected in bonobos (*Pan paniscus*), Cross river gorillas (*G. g. diehli*) and Old World monkeys species) (Liu *et al.*, 2014). Their lack of host specificity and the fact that they include a monophyletic lineage formed by the human *P. vivax* parasites into their diversity, support the idea that the human *P. vivax* developed from within a *Plasmodium*

species which also infected African apes, and that all present human *P. vivax* parasites originated from an ancestor which spread out of Africa (Liu *et al.*, 2014).

P. ovale and *P. malariae*-like parasites were confirmed to be naturally hosted by wild living chimpanzees (*P. t. troglodytes*, *P. t. ellioti* and *P. t. verus* for *P. ovale* and *P. t. troglodytes*, *P. t. verus* for *P. malariae*) (Kaiser *et al.*, 2010; Liu *et al.*, 2010). In the same way as for *P. vivax* and *P. simium*, a genetically highly similar parasite to *P. malariae*, *P. brasilianum*, also infects New World monkeys, which points towards a recent host transfer between New World monkeys and humans (Escalante *et al.*, 1995; Tazi and Ayala, 2011). Larger scale investigations of *P. malariae* and *P. ovale*-like infections in wild NHP, together with phylogenetic analyses, are still missing and will be needed to clarify the evolutionary relationship of these parasites with human strains.

1.1.1.3 Prevalence of malaria parasite infection in wild African great apes

Before large scale screenings on wild ape populations were carried out, the natural prevalence of malaria parasites in some chimpanzee and gorilla populations was suspected to be high, given the frequent detection in sporadic blood or tissue samples (Kaiser *et al.*, 2010; Rich *et al.*, 2009), as well as faecal samples (Krief *et al.*, 2010; Prugnolle *et al.*, 2010) from wild living chimpanzees (all four subspecies) and western gorillas. This was confirmed when prevalence levels of *Plasmodium* spp. infection in wild apes were estimated using detection rates in a broad set of faecal samples (N = 2,739) collected throughout central Africa (Liu *et al.*, 2010). Parasites were found to be endemic in Nigeria–Cameroon chimpanzees (*P. t. ellioti*), central (*P. t. troglodytes*) and eastern (*P. t. schweinfurthii*) chimpanzees, and western lowland gorillas (*G. g. gorilla*), with estimated prevalence levels ranging from 32% to 48%. The estimated rates were obtained after correcting faecal detection rates for the sensitivity of the diagnostic test (in turn estimated by the proportion of positive samples from positive individuals sampled several times in one same day), sample degradation and redundant sampling, but most certainly remain underestimates (Liu *et al.*, 2014, 2010). Another relatively large scale study performed in Gabon on samples from wild living *P. t. troglodytes* and *G. g. gorilla* (N = 1,261) reported faecal detection rates of 15.42 and 21.28%, respectively (Boundenga *et al.*, 2015).

The vast majority of parasites harboured by AGA belong to the *Laverania* clade and *P. vivax*, *ovale* and *malariae*-like strains are more rarely found (Boundenga *et al.*, 2015; Kaiser *et al.*, 2010; Liu *et al.*, 2010). The specific prevalence of *P. vivax*-like parasites in central (*P. t. troglodytes*) and eastern (*P. t. schweinfurthii*) chimpanzees and western (*G. g. gorilla*) and eastern lowland gorillas (*G. b. graueri*), was estimated by Liu *et al.* (2014) during a broad scale screening and ranged from 4 to 8% (close to infection rates in endemic human populations). This also most probably represents an underestimate due to the lower sensitivity of faecal testing compared to blood testing (Liu *et al.*, 2014).

It seems that co-infection with two or more *Plasmodium* parasite species is prevalent (Krief *et al.*, 2010; Liu *et al.*, 2010) (e.g. 55% of positive chimpanzee samples tested by Liu *et al.* (2010)).

Some chimpanzee and gorilla communities are found to be negative for malaria parasites (Boundenga *et al.*, 2015; Liu *et al.*, 2014, 2010). This could be the result of the relatively low sensitivity of faecal detection methods combined with small sample size (Liu *et al.*, 2014). However, low prevalence or absence of malaria parasites in certain communities could also be explained by environmental factors, leading, for example, to a scarcity of efficient vectors, or demographical factors such as variations in host density (see chapter 1.1.2)

1.1.2 Ecology of great ape malaria parasites

1.1.2.1 Vectors

It is well established that mosquitoes are the vectors responsible for transmission of malaria between humans. These vectors belong to the *Anopheles* genus (*An.*), of which approximately 40 species (out of 430) are thought to be competent for malaria parasite transmission (CDC, 2012). They have been studied extensively and mapped in detail (Sinka *et al.*, 2010). The ecology of mosquito species, including habitat and host preferences, differs and determines their importance as vector in given habitats (Sinka *et al.*, 2010). For instance, *An. gambiae* is considered as one of the most efficient vectors in open landscape in sub-saharan Africa (Coetzee, 2004; Sinka *et al.*, 2010). On the other hand, *An. moucheti*, also

highly anthropophilic, is entirely restricted to forested habitat and considered a significant vector of human malaria in this type of environment (Sinka *et al.*, 2010).

AGA malaria vectors have only been researched sporadically so far and current knowledge is still very poor. However, this topic has been raised increasingly due to the concern of the possible zoonotic transfer of malaria parasites from NHP to humans and thus the importance of identifying species that can act as bridges (Verhulst *et al.*, 2012).

Early experiments have shown the susceptibility of some known human malaria vector species to *P. reichenowi* (Blacklock and Adler, 1922; Collins *et al.*, 1986), for a notable exception being *An. gambiae*. Bray (1958), however, managed to infect *An. gambiae* with the formerly called *P. schwetzi,* but the low levels of sporozoites in the salivary glands suggested that these are not the natural vectors. Another experiment actually succeeded in infecting humans with *P. schwetzi* via *Anopheles* mosquito (*An. balabacensis*) bites (Contacos *et al.*, 1970).

So far only two studies have searched specifically for mosquito species responsible for the transmission of malaria parasites between chimpanzees or gorillas in their natural habitat. The first one tested female *Anopheles* (N = 100) captured at chimpanzee (*P. t. schweinfurthii*) natural nesting and feeding sites in Uganda but did not detect any *Plasmodium* parasites (Krief *et al.*, 2012). Failure of detection could be linked to the small number of specimens tested, considering that frequency of detection of *Plasmodium* in mosquitoes is generally low (Krief *et al.*, 2012). The second study, which screened a larger number of female *Anopheles* (N = 1,070) captured in areas hosting AGA populations in Gabon, found DNA from *P. praefalciparum* in one *An. moucheti* and from *P. vivax*-like parasites in one *An. moucheti* and one *An. vinckei* (Paupy *et al.*, 2013). Although this suggests that these mosquito species might support natural transmission of certain AGA *Plasmodium* species, further investigation and confirmation of their role as vectors is necessary. In addition, the dominant *Anopheles* species captured in both these studies differs, suggesting a variability of vectors in function of the investigated geographical sites (**Table 1.1**). In the same line, mosquito trapping performed in and around the forest of the TNP in Côte d'Ivoire to study the distribution of mosquito genera in various habitats, from human settlements to primary forest, only identified one single *Anopheles* in the primary forest (at a camp site), whereas they were abundant in the neighbouring villages (Junglen *et*

al., 2009). Mosquitoes of the *Uranotaenia* genus, on the other hand, were dominant in the primary forest but rarely found in other areas. This also rather suggests that malaria vectors responsible for the transmission of malaria parasites in the resident chimpanzee population differ from those for human parasites.

Other mosquito genera as well as other dipterans than mosquitoes should not be excluded as possible vectors. Biting midges (genus *Culicoides*) for example, transmit *Hepatocystis* sp., which are bat and monkey malaria parasites (Blanquart and Gascuel, 2011), and are known to be abundant in the TNP (personal observation).

Table 1.1 Predominant mosquito species captured in forested habitats of African great apes known to host malaria parasites

Field site	**Mosquitoe species**	**reference**
Park of La Lékédi, Gabon	*Anopheles moucheti* *Anopheles marshallii*	Paupy *et al.* 2013
National Park of La Lopé, Gabon	*Anopheles carnevalei*	Paupy *et al.* 2013
Kibale National Park, Uganda	*Anopheles implexus* *Anopheles vinckei* *Anopheles demeilloni*	Krief *et al.* 2012
Taï National Park, Côte d'Ivoire	*Uranotaenia sp.* (primary forest) *Aedes sp.* (secondary forest)	Junglen *et al.* 2009

1.1.2.2 Extrinsic determinants

The effect of extrinsic factors which could influence exposure to malaria parasites and thus frequency of infection in specific AGA communities, such as environmental factors (habitat type, climate), group size/host density or even choice of sleeping sites, is still largely unknown.

There is some information on host group size and sleeping site in New World primates. In contrast to the encounter-dilution effect which predicts a decreased risk of exposure to an infected vector with increasing group size (Krebs *et al.*, 2014; Mooring and Hart, 1992), malaria prevalence in New World primates was found to increase with average sleeping group size, suggesting that malaria might be a significant cost associated with larger group

sizes (Davies *et al.*, 1991; Nunn and Eckhard, 2005). However, data on primate malaria and group size is still too scarce to draw any firm conclusions. Sleeping site selection in these primates also seems to play a role in malaria infection rates by reducing the risk of attack by the vector (Nunn and Eckhard, 2005). In chimpanzees, two studies looked at malaria and sleeping sites indirectly by capturing mosquitoes at nest sites. While one failed to detect any patterns of association between the choice of nest site and mosquito densities (Koops *et al.*, 2012), the second study found patterns compatible with mosquito avoidance (Krief *et al.*, 2012). Chimpanzees seemed to choose nest sites in drier and higher places in comparison to their feeding sites and these sites were indeed characterized by lower densities of *Anopheles* mosquitoes. Mosquitoes were also less abundant in sites with more nests (Krief *et al.*, 2012).

The influence of the monthly rainfall on the probability of *Plasmodium* detection in chimpanzee and gorilla faecal samples was tested for in one study, but no significant effect was found (Boundenga *et al.*, 2015).

1.1.2.3 Inter-species transmission

Since the discovery of AGA malaria parasites, the marked similarities they share with those infecting humans have raised the questions whether exchange of these parasites between AGA and humans could occur, experimentally and/or naturally.

Early experiments involving captive chimpanzees and humans (volunteers, and/or neurological patients for therapeutic purposes (Contacos *et al.*, 1970; Rodhain and Dellaert, 1943; Rodhain and Dellaert, 1955; Rodhain, 1940)) demonstrated that, for some strains, infection of humans with chimpanzee parasites or vice versa is possible. This was the case for the chimpanzee-borne *P. rodhaini* (probably a *P. malariae*-like strain) (Bray, 1963; Rodhain and Dellaert, 1943; Rodhain, 1940) and the human-borne *P. malariae* (Bray, 1963, 1960; Garnham *et al.*, 1956), as well as for the chimpanzee-borne *P. schwetzi* (probably *P. ovale* or *vivax*-like strains) (Bray, 1963; Coatney, 1968; Contacos *et al.*, 1970; Rodhain and Dellaert, 1955) and the human-borne *P. vivax* (Bray, 1963; Garnham *et al.*, 1956; Rodhain, 1939). In contrast, attempts of transferring the *Laverania* species *P. reichenowi* from chimpanzees to humans failed (Blacklock and Adler, 1922; Rodhain, 1939). This marked host

specificity might be due to the human loss of the common primate Sia N-glycolylneuraminic acid, which is the preferred target of *P. reichenowi* for erythrocyte binding, as opposed to *P. falciparum* which prefers the precursor of the N-glycolylneuraminic acid (the Sia N-acetylneuraminic acid) (Martin *et al.*, 2005). Also, transfer of *P. falciparum* to chimpanzees seemed to be only possible in splenectomised individuals or limited to the liver or to a very low parasitaemia in intact individuals (Bray, 1963; Taylor *et al.*, 1985).

Natural (non-experimental) transmission of certain human malaria parasites to AGA also seems to occur. De facto, early experimental attempts of infecting chimpanzees with *P. falciparum* gave poor results, but captive bonobos (Krief *et al.*, 2010) and chimpanzees (Duval *et al.*, 2010) living in areas of high human *P. falciparum* endemicity have been found to be infected. These infections were probably acquired from humans (Rayner *et al.*, 2011). There is however no information on the viability of such infections and thus the potential for vector-based transmission between apes. Similarly, *P. malariae*, which, so far, has never been detected in wild bonobos, was found in captive individuals (Krief *et al.*, 2010), and a *P. ovale* strain, identical to the human *P. ovale* variant type, was found in a captive chimpanzee (Duval *et al.*, 2009).

From the phylogenetic relationships between known malaria parasites, it appears that, historically, ape *Plasmodium* (*P. praefalciparum*) also managed to naturally cross the species barrier at least once (Liu *et al.*, 2010). This, together with some old experimental results reported above, naturally lead to the assumption that natural zoonotic transfers of AGA malaria parasites might still occur, leading to new host switches, or just to regular transmission events in the same way *P. knowlesi* infection in humans originates from regular zoonotic transfer events from Southeast Asian macaques (Lee *et al.*, 2011; Sharp *et al.*, 2013). Two studies, led in Cameroon (Sundararaman *et al.*, 2013) and Gabon (Délicat-Loembet *et al.*, 2015), searched for evidence of zoonotic transfer of ape *Laverania* species by testing large numbers of blood samples (N =1,402 and N = 4,281) from humans living in the vicinity of chimpanzee and gorilla populations with high *Laverania* prevalence rates. No ape parasites (*Laverania* or non-*Laverania*) were detected in these samples, implying that zoonotic transfers might be rare events. Actually, there is evidence that cross-species transmission of the non-*Laverania* species *P. vivax* from wild apes to humans is currently

ongoing. Since wild chimpanzees in west and central Africa were confirmed hosts of *P. vivax*-like strains, it has been hypothesized that wild apes could act as a natural reservoir (Rayner *et al.*, 2011), explaining the occasional infection of travellers despite the low prevalence of *P. vivax* in the human populations of these regions (Broderick *et al.*, 2015; Culleton *et al.*, 2008; Gautret *et al.*, 2001; Mühlberger *et al.*, 2004; Skarbinski *et al.*, 2006). A *P. vivax*-like parasite isolated from a traveller coming back from a central African forest was actually recently shown to cluster with the AGA isolates, outside of the human lineage, confirming the occurrence of cross species transmission (Liu *et al.*, 2014; Prugnolle *et al.*, 2013). This is supported by the fact that *P. vivax*-like parasite DNA was found in an anthropophilic mosquito species, *Anopheles moucheti* (Paupy *et al.*, 2013).

Given their close resemblance with their human counterparts and the old experimental findings, it is likely that, similarly to *P. vivax*, zoonotic transfers of *P. ovale* and *malariae*-like parasites from chimpanzees also take place. It would be necessary to characterize more of these parasites in order to search for evidence of such transmissions (Sundararaman *et al.*, 2013).

Cross-species transmission between AGA and other NHP sharing their habitat is probably exceptional. So far the only published finding showing such potential is *P. praefalciparum* in one captive greater spot-nosed monkey (*Cercopithecus nictitans*) (Prugnolle *et al.*, 2011b). Yet, during a subsequent study which tested blood from a significant number of wild spot-nosed monkey (N = 292) only *Hepatocystis* spp., and one *Plasmodium* spp. previously described in birds and lizards, were found (Ayouba *et al.*, 2012). *P. vivax* has also specifically been searched for in blood samples (N = 998) from 16 Old World monkey species but except for *Hepatocystis* spp., no malaria parasites were found (Liu *et al.*, 2014). Similar results were obtained after screening specimens from 6 monkey species from Uganda (N = 102) (Thurber *et al.*, 2013) and from 3 monkey species in the TNP, Côte d'Ivoire (N = 38) (S. Calvignac-Spencer, personal communication) (**Table 1.2**). *Hepatocystis* represents one of the numerous genera of malaria parasites, like the genus *Plasmodium*, which seems to be paraphyletic with respect to *Hepatocystis* (Martinsen *et al.* 2008) (**Figure 1.2**). *Hepatocystis* parasites comply with a broad pattern of host-specificity, with distinct lineages that specialize on monkeys and bats, respectively (Ayouba *et al.*, 2012; Schaer *et al.*, 2013).

So far there is no published evidence of transmission of *Hepatocystis* spp. to AGA. Moreover, amongst 30 chimpanzee carcasses from the TNP, Côte d'Ivoire, which were tested for malaria parasites, no *Hepatocystis* spp. were detected (S. Calvignac-Spencer, personal communication). Interestingly, however, a liver sample from one of these chimpanzees tested positive for a saurian *Plasmodium* parasite.

Table 1.2. Malaria parasite screening of monkey species living in African great ape habitats

DRC: Democratic Republic of Congo, [1]: malaria parasites detected in at least one sample, [2] Only one sample was found positive for a *Plasmodium sp.*, which was previously found in birds, lizards and bats.

Monkey species	Number of samples screened	Malaria parasites[1]	Country of origin	Reference
Allenopithecus nigroviridis	46	*Hepatocystis*	DRC	Liu *et al.* 2014
Cercocebus agilis	68	*Hepatocystis*	Cameroon, DRC	Liu *et al.* 2014
Cercocebus torquatus	1	None	Cameroon	Liu *et al.* 2014
Cercocebus atys	8	*Hepatocystis*	Ivory Coast	S. Calvignac-Spencer, personal communication
Cercopithecus ascanius	213	*Hepatocystis*	DRC, Uganda	Liu *et al.* 2014, Thurber *et al.* 2013
Cercopithecus cephus	145	*Hepatocystis*	Cameroon	Liu *et al.* 2014
Cercopithecus mitis	1	None	Uganda	Thurber *et al.* 2013
Cercopithecus neglectus	45	*Hepatocystis*	Cameroon, DRC	Liu *et al.* 2014
Cercopithecus nictitans[1]	520	*Hepatocystis* *Plasmodium sp.*[2]	Cameroon	Liu *et al.* 2014, Ayouba *et al.* 2012
Cercopithecus pogonias	31	*Hepatocystis*	Cameroon	Liu *et al.* 2014
Cercopithecus wolfi	55	*Hepatocystis*	DRC	Liu *et al.* 2014
Colobus angolensis	23	*Hepatocystis*	DRC	Liu *et al.* 2014
Colobus guereza	16	*Hepatocystis*	Cameroon, DRC, Uganda	Liu *et al.* 2014, Thurber *et al.* 2013
Colobus polykomos	14	*Hepatocystis*	Ivory coast	S. Calvignac-Spencer, personal communication
Lophocebus albigena	48	*Hepatocystis*	Cameroon, Uganda	Liu *et al.* 2014, Thurber *et al.* 2013
Lophocebus aterrimus	32	*Hepatocystis*	DRC	Liu *et al.* 2014
Mandrillus sphinx	3	None	Cameroon	Liu *et al.* 2014
Miopithecus ogouensis	4	*Hepatocystis*	Cameroon	Liu *et al.* 2014
Papio anubis	23	*Hepatocystis*	Uganda	Thurber *et al.* 2013
Piliocolobus badius	16	*Hepatocystis*	Ivory Coast	S. Calvignac-Spencer, personal communication
Piliocolobus tholloni	73	*Hepatocystis*	DRC	Liu *et al.* 2014
Procolobus rufomitratus	46	*Hepatocystis*	Uganda	Thurber *et al.* 2013

1.1.3 Pathogenicity and intrinsic determinants of malaria in African great apes

Pathogenicity of malaria parasites and intrinsic factors (e.g. genetics, age, physiological state) driving susceptibility to these parasites or to clinical malaria have been very poorly researched in great apes.

No published studies focused on the consequences of malaria infection on wild great apes' health so far, and information from experimental or captive conditions is extremely limited. Observations made during early experiments on the clinical course of malaria in captive chimpanzees (i.e. mainly the lack of noticeable illness behaviour during various infections with human and possibly chimpanzee strains) led Rodhain (Rodhain, 1940, 1936) to believe that, in natural conditions, chimpanzees do not suffer importantly from malaria and that they can be subject to asymptomatic chronic infections lasting several months. On one occasion he reported a double inoculation with *P. falciparum* and *P. vivax*, in a young individual, which had induced a febrile state with loss of appetite but mildly (Rodhain, 1936). The only indications on the durations of infection actually also come from early experimental studies. They seemed to be able to last for several months at least, with durations as long as 5 month recorded for *P. reichenowi* and *P. schwetzi* (Garnham *et al.*, 1956) and over one year for *P. malariae* (Rodhain, 1940).

Reports from sanctuaries or other facilities hosting captive individuals are rare and variable. In Japan two captive chimpanzees were found to be chronically infected with *P. malariae*, without showing any evidence of illness for 30 years (Hayakawa *et al.*, 2009). *P. falciparum* infection of captive bonobos in the Democratic Republic of Congo was also not associated with apparent clinical signs or increased body temperature (Krief *et al.*, 2010). Similarly, a veterinarian from a sanctuary in the Republic of Congo noted that infected chimpanzees did not seem to develop clinical malaria (R. Atencia, personal communication). On the other hand, in a Cameroonian sanctuary, regular cases of chimpanzees and gorillas who exhibited clinical signs likely to be associated with malaria infection and which resolved after antimalarial treatment were observed (J. Kiyang, personal communication). Moreover, a young chimpanzee newly transferred to a sanctuary after 6 years of captivity in an urban setting developed severe anaemia and hyperthermia concomitant with a high *P. reichenowi* parasitaemia (Herbert *et al.*, 2015). This would suggest that certain malaria parasites can,

under certain circumstances, be pathogenic for chimpanzees. However, extrapolating these observations to the wild is difficult as stress related to captivity could also impact on the effect of such infections and whether these individuals were initially naïve to the *Plasmodium* strains they were found infected with (whether human or chimpanzee derived) is undetermined (strains novel to a chimpanzee's immune system would be more likely to cause illness). One publication also reported the death of a 1 year old captive chimpanzee infected with *P. reichenowi*, but given the extremely poor and stressful conditions this individual was kept in, whether malaria effectively played a significant role in the fatal outcome is questionable (Tarello, 2005).

The general impression given by these elements as well as the absence or rarity of reported malaria-like clinical signs or death associated with infection in individuals observed on a sometimes daily basis (wild habituated or captive individuals) (e.g. Kaiser *et al.* (2010)), is that wild living chimpanzees are mostly undisturbed by malaria infections, or, that at least the clinical impact and related discomfort are generally mild enough to be invisible to observers eyes when not specifically searching for these. Also, as pointed out by Rayner *et al.* (2011), the very high prevalence rates of malaria parasite infections observed in numerous wild chimpanzee and gorilla populations indicate that severe outcomes are probably rather rare.

However, perhaps as in humans (Doolan *et al.*, 2009), constant exposure leads to acquired immunity (parasitological and/or clinical) and lightens the burden of the infection in adults, with the hardest consequences falling upon the infants. In humans, pregnant women and immunocompromised people are, like young children, also part of the most vulnerable groups to infection, higher parasitaemia or clinical malaria amongst populations from endemic areas (WHO, 2015).

Increased susceptibility during pregnancy is believed to be the result of a combination of immunological and hormonal changes, together with the ability of *P. falciparum* infected erythrocytes to sequester in the placenta (Rogerson *et al.*, 2007). Malaria during pregnancy can have deleterious consequences for pregnancy outcome and infant health (Desai *et al.*, 2007; Guyatt and Snow, 2001).

Alteration of the immune system is often generated by co-infections with other pathogens. There is evidence of pathological interaction between HIV and *Plasmodium*, HIV-

seropositive people being at increased risk of *Plasmodium* infection and development of clinical malaria (Patnaik *et al.*, 2005; Whitworth *et al.*, 2000). The impact of co-infection with helminths is also widely studied but results are controversial and interactions remain unclear; susceptibility can be influenced in both ways depending on the parasites (Bejon *et al.*, 2008; Efunshile *et al.*, 2015; Faure, 2014; Fernandez-Nino *et al.*, 2012; Kelly-Hope *et al.*, 2006). Many other microorganisms also seem to interact with malaria parasites (e.g. hepatitis B virus, *Mycobacterium tuberculosis*), but mechanisms are generally not clear (Faure, 2014). Important to note is that interactions during co-infections do not only occur via the immune system of the host, but also via shared resource competition (Graham, 2008; Pedersen and Fenton, 2007; Rynkiewicz *et al.*, 2015).

Amongst the intrinsic factors known to influence susceptibility to malaria in humans are also genetic factors which can play a role in conferring a certain degree of resistance to infection or disease. Genetic regulation of the immune response by certain alleles encoding the major histocompatibility complex I and II, some of the interleukines, the CD40 ligand, the nitric oxide synthase and interferon α and γ receptors have been associated with protection against severe malaria (Hill *et al.*, 1991; Kwiatkowski, 2005). Mutations leading to variants of erythrocyte characteristics also provide protection against parasite invasion, like Duffy negativity for *P. vivax*, glycophorin A, B and C deficiency for *P. falciparum*, *Haemoglobin* E (β-globin gene mutation); or protection against clinical malaria, such a *Haemoglobin* S (sickle *haemoglobin*) and C (both β-globin gene mutations), glucose-6-phospate dehydrogenase (G6PD) deficiency and α + Thalassemia (defective production of α globin) (Kwiatkowski, 2005; Williams, 2006). In chimpanzees, the diversity of the G6PD and β-globin genes has been examined but no mutations appear to have been selected for malarial resistance (MacFie *et al.*, 2009; Verrelli *et al.*, 2006). Moreover, Liu *et al.* (2014) sequenced 134 samples from AGA but did not find any Duffy negative phenotype.

Apart from these few genetic analyses, intrinsic determinants of susceptibility to infection, or higher parasitaemia and potentially clinical malaria, have not been researched in AGA.

1.2 Non-invasive investigation of malaria infection

1.2.1 The use of non-invasive samples to study parasites infecting great apes

Faeces, urine and saliva samples have been used routinely for decades to study some of the parasites infecting the gastro-intestinal, urinary and sometimes respiratory systems (such as helminths, protozoa and bacteria) through microscopy or culture. Development of molecular methods has opened a whole new range of possibilities regarding the use of such samples by expanding their use to the detection of all sorts of parasites, including those causing systemic infections.

This can be achieved by amplifying specific fragments of these parasites' genetic material in faecal, urinary or saliva DNA/ribonucleic acid (RNA). The mechanisms by which nucleic acids of some of these parasites end up in faeces, urine or saliva are not always entirely understood, but over the past 15 years this has been proven to occur for many parasites in great apes, such as simian immunodeficiency virus (SIV) (M L Santiago *et al.*, 2003a), hepatitis B virus (Makuwa *et al.*, 2005), adenovirus (Roy *et al.*, 2009), paramyxovirus (Efunshile *et al.*, 2015; Kondgen *et al.*, 2010), polyomavirus (Scuda *et al.*, 2013), *Plasmodium* parasites (Prugnolle *et al.*, 2010) and herpesvirus (Seimon *et al.*, 2015).

Serological methods have also been increasingly and successfully applied to non-invasive samples, e.g. antibodies against simian T-cell leukemia virus and SIV are found in chimpanzee urine and faeces, respectively (Leendertz *et al.*, 2004; M L Santiago *et al.*, 2003b).

These methodological advances were a major leap in the field of AGA pathogen research. Great Apes generally live in habitats difficult to access, mostly in dense forests rendering safe sampling of blood and tissue specimens very challenging and unpractical. In addition, routine immobilization of wild great apes for the sole purpose of research is not advocated for ethical and safety reasons (Gillespie *et al.*, 2008; F H Leendertz *et al.*, 2006). Tissue and blood samples are thus usually only obtained from dead individuals or occasionally during immobilizations justified by the need for a veterinary intervention. The possibility to use non-invasive samples instead, which are by far more easily accessible, especially faecal samples, offers incredible opportunity to study microorganisms in the wild (Calvignac-Spencer *et al.*, 2012; F H Leendertz *et al.*, 2006) and perform large scale epidemiological

surveys, such as for SIV (Keele *et al.*, 2006; Van Heuverswyn *et al.*, 2007) and malaria parasites (Boundenga *et al.*, 2015; Liu *et al.*, 2014, 2010).

1.2.2 Non-invasive samples and malaria detection

Classically, malaria parasites are detected and characterized directly by blood smear microscopy. Detection of circulating malaria antigens by rapid diagnostic tests is also commonly used (Tangpukdee *et al.*, 2009). Nowadays, however, an increasing number of research laboratories rely on polymerase chain reaction (PCR)-based amplification of *Plasmodium* DNA and sequence analysis to detect, diagnose or genotype these parasites in blood samples (de Monbrison *et al.*, 2003; Snounou *et al.*, 1993). The search for alternative, more practical detection methods to those requiring drawing blood led to the discovery that *Plasmodium* DNA can also be amplified from human urine, saliva and faeces (A-Elgayoum *et al.*, 2010; Jirku *et al.*, 2012; Mharakurwa *et al.*, 2006; Nwakanma *et al.*, 2009; Putaporntip *et al.*, 2011). Molecular detection targeting the small subunit ribosomal RNA gene (18s rRNA) in saliva and urine is highly specific (97-100%) but less sensitive (73-84 % for saliva and 32-44 % for urine) compared to blood microscopy (Buppan *et al.*, 2010; Nwakanma *et al.*, 2009), and quantitative PCR results from saliva seem to correlate with blood parasite density (Nwakanma *et al.*, 2009). Shorter DNA fragments are amplified more efficiently as parasite DNA in urine and saliva is probably partly degraded and thus highly fragmented (Mharakurwa *et al.*, 2006). In addition, it seems that targeting mitochondrial DNA (mtDNA) sequences such as the cytochrome b (cyt b) gene might yield higher sensitivity compared to the small subunit ribosomal RNA gene as mtDNA is present in higher copy numbers (Putaporntip *et al.*, 2011).

Prugnolle *et al.* (2010) were the first to try using faecal samples to research *Plasmodium* infection in wild chimpanzees and gorillas and to show the suitability of this non-invasive method. They amplified and sequenced partial sequences of the mitochondrial cyt b gene from faecal DNA. Since then, several studies have been using PCR assays on faecal DNA to investigate malaria parasites diversity in wild AGA, targeting the same gene as well as apicoplast, nuclear or other mitochondrial fragments (Boundenga *et al.*, 2015; Kaiser *et al.*, 2010; Liu *et al.*, 2014, 2010), occasionally performing single genome amplifications (SGA) (Liu

et al., 2014, 2010). *Plasmodium* cyt b gene detection in urine and faeces from macaques was also shown to be effective (Kawai *et al.*, 2014). The probability of *Plasmodium* detection was shown to be higher in fresh faecal samples compared to those collected later than 24 hours post defaecation (Boundenga *et al.*, 2015).

To date, the mechanisms by which malaria parasite DNA is shed in the faeces are not yet entirely clear. The sensitivity of testing faeces compared to blood remains unknown and thus also the extent to which faecal detection rate reflects the actual parasitaemia prevalence.

An experimental study on mice showed that DNA from both erythrocytic and pre-erythrocytic (liver) parasite stages could be found in the faeces, with increased DNA quantities during the erythrocytic cycle, and recommended caution when interpreting results as faecal detection doesn't necessarily constitute proof of blood infection (Abkallo *et al.*, 2014). Similarly, *Plasmodium* DNA was detected in faeces from one experimentally inoculated macaque even when no blood stages were observed, and could be found in faeces for a longer period of time compared to urine and blood (Kawai *et al.*, 2014). The most likely routes through which malaria parasite DNA ends up into the faeces are intestinal bleeding and excretion of the bile, which could contain cell-free DNA from parasites cleared in the liver (Abkallo *et al.*, 2014; Kawai *et al.*, 2014).

If blood infection as well as liver parasite stages can be detected in faeces, an overestimation of blood infection prevalence would be plausible. However, by comparing *P. vivax* PCR results from a set of captive chimpanzee blood and faecal samples (unpaired samples, i.e. blood and faecal samples were not collected together from one same individual), Liu *et al.* (2014) found that faecal detection actually underestimated prevalence of infection. Without access to paired faecal and blood samples though, real status of malaria parasite infection remains unknown, and thus sensitivity of faecal detection cannot be determined. However, as illustrated by two non-invasive epidemiological studies (Liu *et al.*, 2014, 2010), one way to obtain an approximation of faecal PCR sensitivity is to use the proportion of positive PCR samples from apes sampled several times in one same day and with at least one PCR positive sample, assuming the likelihood of complete infection clearing over the course of a single day is very low. This represents, however, an overestimation of the sensitivity as it doesn't take into account affected individuals who always tested (perhaps falsely) negative. Sensitivity approximates were estimated in this manner at 57 %

and 32 % for the *Plasmodium* cyt b and *P. vivax* cytochrome c oxidase subunit I (cox 1) PCR assays used by Liu *et al.* (2010, 2014).

1.3 Conclusions

It is clear that, to date, most of the knowledge on wild AGA malaria is concentrated around parasite diversity, distribution, and phylogenetic relationships with malaria parasites infecting other species, humans in particular. Indeed, the majority of research studies ultimately aim at identifying potential animal reservoirs with ongoing zoonotic transmission as well as assessing the risk of emergence of new malaria strains in the human population (by, inter alia, exploring origins of the current human malaria parasites). Many gaps, however, still need to be filled in order to achieve these objectives, e.g. further exploration of vectors, phylogenetic relationships of ape-derived *P. malariae* and *P. ovale* strains with human strains, and zoonotic infections in humans living close to AGA communities.

This also leaves many questions to address to further understanding of the basic epidemiology and biology of malaria parasites in AGA, which in turn could serve as a model for human malaria, as well as help comprehend the significance of malaria for great ape populations. As a matter of fact, the epidemiological determinants and the effects of malaria infection in wild AGA have been left largely unexplored. What are the influences of intrinsic and extrinsic factors on infection, parasite density or perhaps, disease? Are there visible patterns of infection in specific populations? How frequently and for how long are individuals infected? What are the pathological consequences of malaria infection? Does infection have a significant impact on great ape's health? Or could the impact of malaria be sufficiently subtle to be misleading, just like SIV infection in chimpanzees, for many years believed to be rather trivial for their health (non-pathogenic), but whose actual deleterious impact on health only became apparent recently when examined at the level of an entire community (Keele *et al.*, 2009; Rudicell *et al.*, 2010)? Does malaria play a role in sociality, demographics, population dynamics and general fitness? Are there any indications of selective pressure exerted by malaria on AGA evolution or development of resistance? These are just a fraction of the multiple questions that still need to be addressed. Non-invasive methods to study pathogen infections as well as monitor health parameters (e.g. body

temperature, inflammation markers, hormone levels) are now available, rendering attempts to answer some of these questions actually conceivable. In the meantime, further exploration, improvement and testing of non-invasive methods needs to be pursued in order to enhance sensitivity, facilitate interpretation of results and broaden research possibilities.

OBJECTIVES OF THE STUDY

The research performed in the frame of this doctoral work constitutes the first part of a broader study aimed at exploring the determinants and effects of malaria infection in AGA. Within this framework, a logical way to start with is by focusing on some of the major intrinsic factors that might determine susceptibility to infection, and perhaps illness, allowing a first comparison with epidemiological features in humans, and also the identification of targets best suited to continue further investigation.

The specific objectives set for this study were the following:

I. - Investigate the effects of age and pregnancy, two well-known factors of influence for human malaria, on malaria infection in a wild chimpanzee community
   - Achieve this by applying for the first time non-invasive molecular methods to investigate such type of epidemiological questions

II. - Contribute to the development of non-invasive methodology by investigating the effect of predation on the risk of contamination of predator faecal samples with microorganisms infecting prey

To accomplish this, the practical aims were to collect samples and data from a wild chimpanzee community in the TNP, Côte d'Ivoire, habituated by the TCP (Department of Primatology, MPI, Leipzig) and to carry out laboratory analysis of these samples, as well as samples from a large TCP sample bank, at the RKI (Research Group Epidemiology of Highly Pathogenic Microorganisms, Berlin).

CHAPTER 2

AGE-RELATED EFFECTS ON MALARIA PARASITE INFECTION IN WILD CHIMPANZEES

Based on the manuscript: De Nys HM, Calvignac-Spencer S, Thiesen U, Boesch C, Wittig RM, Mundry R, Leendertz FH. 2013. Age-related effects on malaria parasite infection in wild chimpanzees. Biology Letters 9(4):20121160.

2.1 Introduction

The development of molecular diagnostic tools and the use of non-invasive faecal samples have recently prompted broad-scale investigations of the malaria parasite (*Plasmodium* spp.) infections in wild AGA (Kaiser *et al.*, 2010; Liu *et al.*, 2010). This has notably led to the discovery of at least six distinct parasite species infecting wild chimpanzees (*Pan troglodytes*). Three of them, *P. reichenowiet al.*, *P. gaboni*, and *P. billcollinsi* are part of the *Laverania* subgenus, to which *P. falciparum*, the species responsible for malignant malaria in humans also belongs (Prugnolle *et al.*, 2011a; Rayner *et al.*, 2011); they are by far the most frequently encountered (Liu *et al.*, 2010). The three other species are respectively related to the human-infecting *P. vivax*, *P. ovale* and *P. malariae* parasites and occur more rarely (Kaiser *et al.*, 2010; Liu *et al.*, 2010).

While the genetic diversity of chimpanzee malaria parasites has now been extensively studied in the wild, substantially less is understood about the epidemiological drivers of infection. Important in this context are the virulence of the parasite – i.e., the added mortality due to infection – and age-related variation in prevalence. In human populations, for example, parasite prevalence and malaria related morbidity/mortality decrease with age, which reflects the progressive mounting of a protective immunity (Doolan *et al.*, 2009; WHO, 2015).

Here, we present a cross-sectional study which addresses the age-distribution of malaria parasite infection in a group of wild chimpanzees.

2.2 Methods

2.2.1 Study site and population

This study was carried out on a wild western chimpanzee (*P. t. verus*) population in the TNP (3300 km^2), located in the south-west of Côte d'Ivoire (5°50'N, 7°21'W). The TNP is constituted of a primary tropical forest, which hosts a large diversity of mammals, including chimpanzees and 11 species of monkeys (UNESCO, 2015). It was classified as a UNESCO World Heritage Site in 1982. The climate is characterized by two rainy seasons (March-June and September-October) and two dry seasons (July-August and November-February)

(Boesch and Boesch-Achermann, 2000). The annual rainfall and average temperature are 1800 mm and 24°C, respectively (Anderson *et al.*, 2005).

The chimpanzee population of the TNP was estimated at 264 weaned individuals in 2012 (WCF, 2012). This population has been subjected to behavioural research since 1979, when the TCP was founded (MPI, Leipzig) (Boesch and Boesch-Achermann, 2000). Currently, the project works with three neighbouring communities of chimpanzees which have been habituated to human presence for research purposes. A chimpanzee health project has also been developed since 2001, in collaboration with the RKI, Berlin, resulting in the regular collection of non-invasive samples from habituated individuals (F H Leendertz *et al.*, 2006). So far, two studies have investigated the presence of malaria parasites in these chimpanzees by testing a limited number of tissue and blood samples collected from individuals who had died from disease or other causes (N = 10 in Rich *et al.* (2009); and N = 16 in Kaiser *et al.* (2010)) and confirmed the presence of *P. reichenowi*, *P. gaboni*, and *P. vivax*, *ovale* and *malariae*-like parasites. Kaiser *et al.* (2010) was also able to obtain two *Plasmodium* sequences from a small set of faecal samples (N = 30).

2.2.2 Sample collection

One hundred forty one faecal samples were collected from seven female and 12 male chimpanzees belonging to an habituated community of 25 individuals (Boesch and Boesch-Achermann, 2000), in which malaria parasites are known to circulate (Kaiser *et al.*, 2010; Rich *et al.*, 2009). Faecal samples were collected within 5 minutes after defecation using single-use plastic bags, transported to camp in a cool box within 12 hours and then stored in liquid nitrogen until shipment to Germany. On average, 7.42 samples per individual were collected over two time periods (October-November 2011, average 2.94; March-April 2012, average 4.88). Ages at time of sampling ranged from 3 to 47 years.

2.2.3 Molecular biology

DNA was extracted from pea-sized faecal samples using EURx Gene MATRIX Stool DNA Purification Kit (Roboklon, Berlin, Germany). Total DNA was measured by fluorometry,

using a Qubit (Invitrogen, Carlsbad, CA). Content in mammal mtDNA was assessed using a quantitative PCR (qPCR) assay targeting a 200 base pair (bp)-long fragment of the 16S gene (16Smam1/2; primers and conditions described in Calvignac-Spencer *et al.*, 2013).

For malaria parasite detection, a nested PCR assay was performed. Two microliters (µL) of DNA extract were used to seed a first standard PCR reaction targeting a 170 bp-long fragment of malaria parasite DNA in the non-coding region of the mtDNA (qPlasm1f 5'-CTGACTTCCTGGCTAAACTTCC-3' and qPlasm1r 5'-CATGTGATCTAATTACAGAAYAGGA-3'). This PCR assay was performed in a total volume of 25 µL and contained 0.2 micromolar (µM) of each primer, 200 µM deoxynucleoside triphosphates (dNTP), 4 millimolar (mM) MgCl2, 2,5 µl 10X PCR buffer and 0.25 µl Platinum® Taq polymerase (Invitrogen). The reaction was run under the following conditions: 3 minutes (min) at 95°C, 15 cycles [1 min at 95°C, 1 min at 54°C, 1 min at 72°C], 7 min at 72°C. Using 1 µL of this first reaction, a nested qPCR targeting a 90 bp-long fragment (qPlasm2f 5'-AGAAAACCGTCTATATTCATGTTTG-3' and qPlasm2r 5'-ATAGACCGAACCTTGGACTC-3') was then performed using GoTaq® qPCR Master Mix (Promega, Fitchburg, WI) under the following cycling conditions: 5 min at 95°C, 40 cycles [15 s at 95°C, 30 s at 57°C, 30 s at 60°C], ramp increase from 55 to 95°C (for dissociation analysis). The qPCR was used for screening purposes only; quantification of malaria parasite DNA in the initial DNA extracts was not possible given that the qPCR was run on previously amplified products.

Positive samples were then subjected to a semi-nested PCR targeting a longer malaria parasite mtDNA fragment (350 bp) comprising the 3' end of the cox 1 gene, a short intergenic region and the 5' end of the cyt b gene. In the first round primers Plasmseq1f 5'-GGATTTAATGTAATGCCTAGACGTA-3' and Plasmseq1r 5'-ATCTAAAACACCATCCACTCCAT-3' were used; in the second round Pspcytbf1 5'-TGCCTAGACGTATTCCTGATTATCCAG-3' [1] and Plasmseq1r. The first reaction was run from two µL of DNA extract while the second was seeded with two µL of the first round reaction. PCR assays were performed in a total volume of 25 µL and contained 0.2 µM of each primer, 200 µM dNTP (with deoxyuridine triphosphate (dUTP) replacing deoxythymidine triphosphate (dTTP) in the first round), 4 mM $MgCl_2$, 2,5 µl 10X PCR buffer and 0.25 µl Platinum® Taq polymerase (Invitrogen). Both PCRs were run under the following conditions: 3 min at 95°C, 50 cycles [40 s at 95°C, 45 s at 57°C, 45 s at 72°C], 7 min at 72°C.

PCR products were sequenced on both strands according to Sanger's methodology. Sequences were analysed using Geneiousv5.4 (Kearse *et al.*, 2012) and compared to publicly available sequences using BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990). All sequences identified in this study exhibited 99 to 100% sequence identity to published sequences, which allowed for unambiguous species assignment. Sequences were deposited in EMBL (European Molecular Biology laboratory) under accession numbers HF952925-HF952956.

Of note, the PCR systems described here were newly designed and were therefore first validated on a broad range of malaria parasite positive and negative samples (data not shown).

2.2.4 Statistical analyses

To investigate what influenced the probability of a positive PCR test for *Plasmodium* spp., we used a Generalized Linear Mixed Model (GLMM; (Baayen, 2008)) with binomial error structure and logit link function. Into this we included age and sex, defaecation time and defaecation time squared as fixed effects and the individual identity (ID) as a random effect. The rationale for including defaecation time squared into the model was that it seemed possible that the response showed a non-linear diurnal pattern. We z-transformed defaecation time and age to a mean of zero and a standard deviation of one. To control for differences in the amount of material used for analyses, we included it as an offset term into the model. However, we had two measures of the amount of material analysed (total_DNA and mammal_mt_DNA) and, hence, ran two models, one with each of the two included as an offset term (after log-transforming).

The probability of a positive result seemed likely to be temporally auto-correlated beyond what could be explained by the model. To control for auto-correlation, we explicitly incorporated it into the model, using an approach identical to that described in Furtbauer *et al.* (2011). To determine whether the period of sampling (October-November 2011 or March-April 2012) had any effect on the models, two alternative models that included it as a random effect were also tested.

To determine the significance of age and sex, the two predictors we were mainly interested in, we compared the fit of the full model with that of a null model lacking these

two fixed effects, but comprising all other terms present in the full model (Forstmeier and Schielzeth, 2011) using a likelihood ratio test (Dobson, 2002).

To rule out the possibility that the results were largely dependent on certain individuals we excluded each individual once from the data and repeated the analysis. The estimated coefficients were similar to the ones derived from all data. Hence, the results were not unduly driven by certain individuals. The model was fitted in R (version 2.14.1; R Development Core Team 2012) using the function lmer of the package lme4 (Bates *et al.*, 2013) and the autocorrelation term was derived using an R-script written by R.M. and using the R-function optimize. Likelihood ratio tests were conducted using the R-function ANOVA with the argument 'test' set to 'Chisq'.

2.3 Results

From a nested qPCR assay, *Plasmodium* spp. sequences were detected in 35% (N = 50) of the samples. All individuals but three (84%) were found to be positive at least once over the course of this sampling scheme (*ca.* two months; **Figure 2.1**). Malaria parasite presence was detected in at least one member of the group on 31 days out of the 43 (72%) days during which samples were acquired. Days for which no malaria parasite could be detected were also those less intensively sampled (for 'positive' days: mean four samples and standard deviation 1.9; for 'negative' days: mean 1.25 sample and standard deviation 0.4).

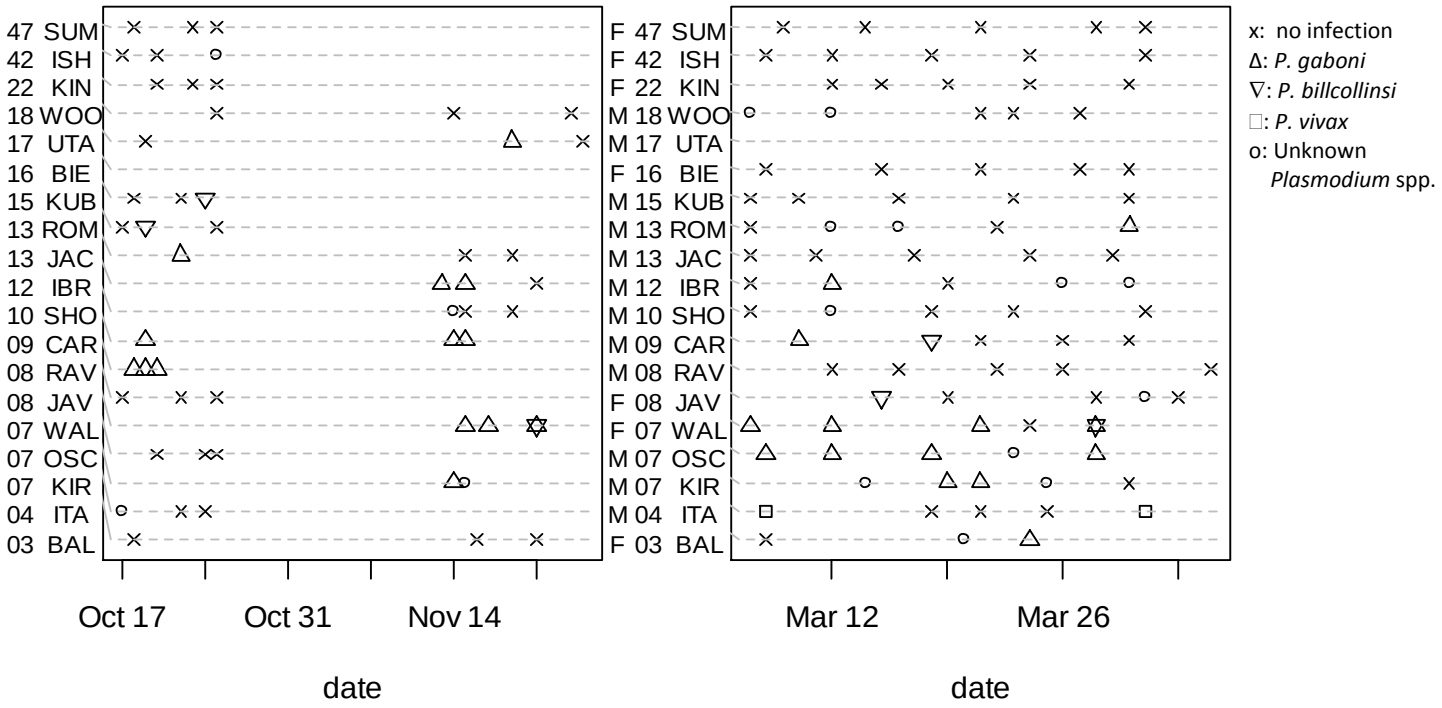

Figure 2.1. Malaria parasite infection through time

Each line represents an individual (identity is given by the three letter code) and symbols represent faecal samples collected and tested. Individuals are ordered according to their age, which is shown next to individual short names. The sex of each individual is indicated by F for females and M for males.

Overall, a full model including an effect of age and sex on the probability of a positive result was preferred to a null model not accounting for the effect of these variables (likelihood ratio test comparing full and null model, model with total_DNA as offset term: χ^2 = 11.44, d.f. = 2, p = 0.003; model with mammal_mt_DNA: χ^2 = 12.2, d.f. = 2, p = 0.002). However, detailed inspection of individual results revealed that only age and the autocorrelation term were significant (sex of animal and time of day were not; **Table 2.1** and **Table 2.2**).

Table 2.1. Results of GLMM model with total_DNA as the offset term

There was no overall effect of day time (likelihood ratio test comparing the full model with a reduced model lacking day time and day time^2: χ^2 = 1.52, d.f. = 2, p = 0.468). Age, day time and the autocorrelation term were z-transformed (s.e: standard error).

Term	estimate	s.e.	z	p
Intercept	-0.923	0.575	-1.606	0.108
Age	-1.317	0.515	-2.557	0.011
sex (0=F, 1=M)	0.463	0.572	0.808	0.419
day time	0.238	0.217	1.098	0.272
day time^2	-0.120	0.218	-0.550	0.582
autocorrelation term	0.523	0.192	2.726	0.006

Table 2.2. Results of GLMM model with mammal_mt_DNA as the offset term

There was no overall effect of day time (likelihood ratio test comparing the full model with a reduced model lacking day time and day time^2: χ^2 = 0.91, d.f. = 2, p = 0.634). Age, day time and the autocorrelation term were z-transformed.

term	estimate	s.e.	z	p
intercept	-1.193	0.543	-2.197	0.028
age	-1.103	0.453	-2.433	0.015
sex (0=F, 1=M)	0.760	0.545	1.395	0.163
day time	0.212	0.222	0.956	0.339
day.time^2	0.030	0.223	0.135	0.893
autocorrelation term	0.703	0.202	3.482	0.000

A clear decrease of the detection probability with age was observed for both models (model with mammal_mt_DNA: estimate + s.e. = -1.10 + 0.45, z = -2.43, p = 0.015; model with total_DNA as offset term: estimate + s.e. = -1.32 + 0.51, z = -2.56, p = 0.011; **Figure 2.2**).

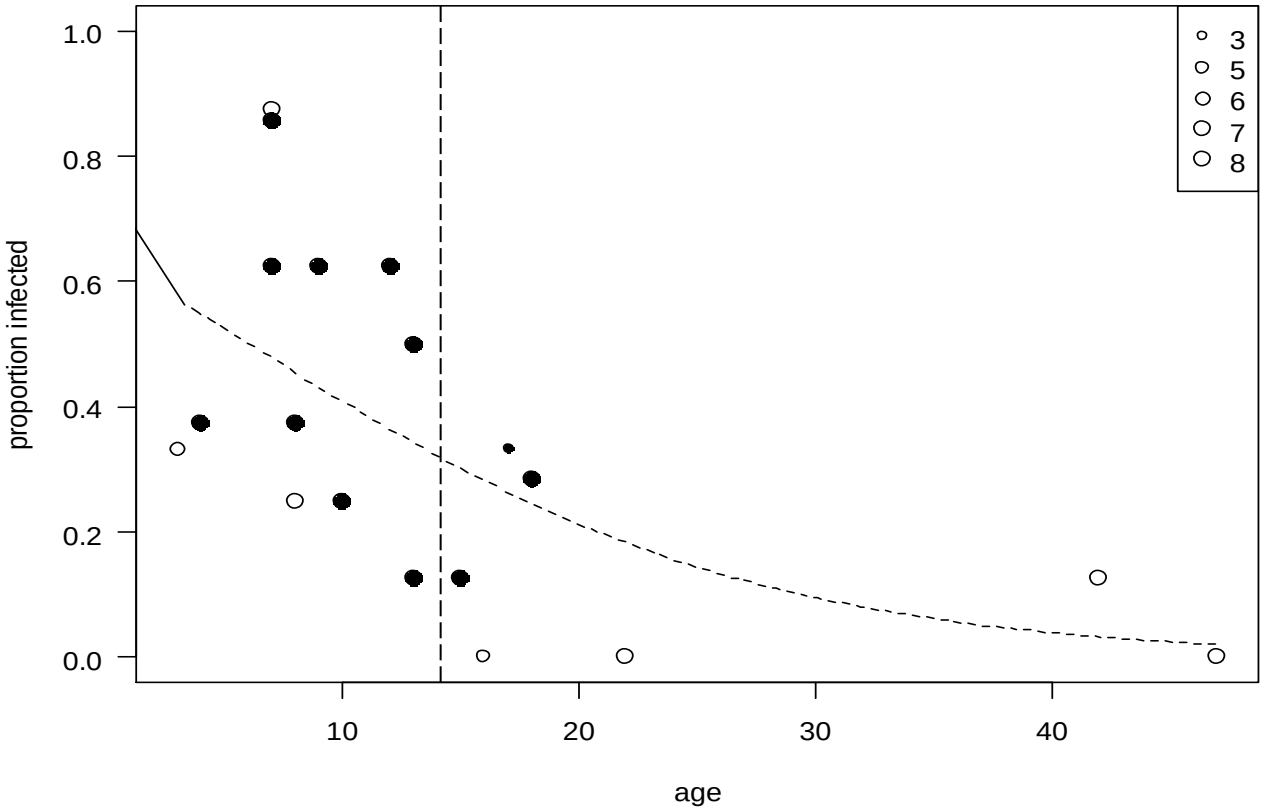

Figure 2.2. Malaria parasite infection frequency as a function of age

Circles represent individuals. Circle areas are proportional to the number of samples analysed for a given individual; female are shown using open circles, males using filled circles. The vertical dashed line represents the age of maturity in female chimpanzees in Taï (14y; [7]). Offset term = mammal_mt_DNA.

Models including sampling time as a random effect yielded essentially the same results (**Table 2.3** and **Table 2.4**).

Table 2.3. Results of GLMM model with total_DNA as the offset term and sampling period included as a random effect

Full reduced model comparison: $\chi 2 = 11.44$, d.f. = 2, p = 0.003. Age and day time were z-transformed.

term	estimate	s.e.	z	p
intercept	-0.923	0.575		
age	-1.317	0.515	-2.557	0.011
sex (0=F, 1=M)	0.463	0.572	0.808	0.419
day time	0.238	0.217	1.098	0.272
day.time^2	-0.120	0.218	-0.550	0.582
autocorrelation term	0.523	0.192	2.726	0.006

Table 2.4. Results of GLMM model with mammal_mt_DNA as the offset term and sampling period included as a random effect

Full reduced model comparison: $\chi 2 = 12.2$, d.f. = 2, p = 0.002). Age and day time were z-transformed.

term	estimate	s.e.	z	p
intercept	-1.193	0.543		
age	-1.103	0.453	-2.433	0.015
sex (0=F, 1=M)	0.760	0.545	1.395	0.163
day time	0.212	0.222	0.956	0.339
day.time^2	0.030	0.223	0.135	0.893
autocorrelation term	0.703	0.202	3.482	<0.001

For most positive samples (N = 34), it was also possible to determine informative sequences. This revealed a clear predominance of *P. gaboni*, which was identified in 82% of the positive samples. *P. billcollinsi* and *P. vivax* were, however, also detected in 18 and 6% of the positive samples, respectively (two cases of co-infection with *P. gaboni* and *P. billcollinsi* were detected). Although most series of positive samples involved a single species, two cases of an apparent switch from one parasite species to another were observed (CAR in March and WAL in November; **Figure 2.1**). The failure in obtaining sequences for some of the positive samples (N = 17) was likely due to faecal DNA fragmentation resulting in insufficient quantities of the larger 350 bp fragment targeted for sequencing.

The same GLMM model was run again after excluding positive samples for which no sequences were obtained (N = 17) and gave similar results: a full model including an effect of age and sex on the probability of a positive result was preferred (likelihood ratio test comparing full and null model, model with total_DNA as offset term: $\chi^2 = 12.41$, d.f. = 1, $p < 0.001$) and there was a clear decrease of the detection probability with age (estimate + s.e. = -2.13 + 0.78, z = - 2.73, p =0.006; **Table 2.5**).

Table 2.5. Results of GLMM model with dataset excluding non-sequenced positive samples
Mammal_mt_DNA is set as the offset term. Age, day time and the autocorrelation term were z-transformed.

term	estimate	s.e.	z	p
intercept	-2.278	0.776	-2.936	0.003
age	-2.132	0.782	-2.728	0.006
sex (0=F, 1=M)	0.719	0.639	1.125	0.261
day time	0.543	0.306	1.772	0.076
day time^2	0.008	0.283	0.030	0.976
autocorrelation term	0.868	0.247	3.512	<0.001

2.4 Discussion

The general detection rate was 35% overall, which is well in line with previous estimates in wild chimpanzees (Kaiser *et al.*, 2010; Liu *et al.*, 2010). However, almost every individual chimpanzee was found positive at least once in the course of this short study. Taking into account sampling intensity, our data also suggest that at every point in time at least one individual of this group of 25 wild chimpanzees is infected. Given the low chimpanzee density in the region (Boesch and Boesch-Achermann, 2000), it appears likely that circulation of malaria parasites is mainly supported by transmission within groups. Because these groups are, in general, quite small, it can be hypothesized that malaria parasites are locally (within groups) exposed to intense genetic drift. This could end up with group-specific infection with particular parasite species and/or shift of locally dominant parasite species through time, particularly when chimpanzee group size decreases.

Although negative samples do not necessarily mean the absence of infection, but possibly low parasitaemia and shedding in the intestinal lumen, the age-related decrease of the detection probability of *Plasmodium* spp. most probably reflects a decline in parasite prevalence and/or parasite density with age, as has been observed in human populations from endemic areas (Doolan *et al.*, 2009; WHO, 2015). To our knowledge, this is the first indication that epidemiological characteristics of malaria parasite infection in wild chimpanzee populations might be comparable to those in human populations.

As in humans (Doolan *et al.*, 2009), the development of acquired immunity probably plays an important role in explaining the observed patterns. Throughout this process, malaria parasites might also contribute to directly decimating young chimpanzees. Histopathological findings and molecular analyses performed on more than 30 dead adult chimpanzees from the same area and community do not support a marked pathogenicity of malaria parasites in adults, since malaria was thus far never identified as a possible cause of death (Kaiser *et al.*, 2010). For young chimpanzees, however, the question remains completely open. While it is known that mortality in young chimpanzees is high (Hill *et al.*, 2001), their bodies are rarely accessible, either because they have less chances to be found opportunistically or because their carcasses are carried for several days by their mothers (data not shown). This makes the determination of young chimpanzees' cause of death a

tricky issue and the involvement of malaria parasites currently cannot be ruled out. Even though at this stage, we cannot pinpoint pathogenicity of malaria parasites found in wild chimpanzees, our results suggest a continuous exposure of this population, leading to the development of a resistance to infection and/or high parasitaemia.

CHAPTER 3

MALARIA PARASITE INFECTION AND PREGNANCY IN WILD CHIMPANZEES

Based on the manuscript: De Nys HM, Calvignac-Spencer S, Boesch C, Dorny P, Wittig RM, Mundry R, Leendertz FH. 2014. Malaria parasite detection increases during pregnancy in wild chimpanzees. Malaria Journal 13(1):413.

3.1 Introduction

Chimpanzees, gorillas and humans are hosts to a similar diversity of malaria parasites (*Plasmodium* spp.) (Kaiser *et al.*, 2010; Liu *et al.*, 2010). The phylogenetic relationships of African great ape (AGA) *Plasmodium* spp. have been described in detail but many aspects of their biology are poorly understood. For example, while the high prevalence of the infection in wild AGA and the absence of recorded death imputable to these parasites points at a weak pathogenicity (De Nys *et al.*, 2013; Rayner *et al.*, 2011), the fact that all *Plasmodium* spp. infecting humans cause morbidity and that some experimental infection of chimpanzees evidenced malaria-like symptoms (Rodhain, 1936) raises the possibility of some impact on wild AGA population health.

In fact, even the basic features of the epidemiology of AGA malaria parasites are only beginning to be understood. A naturally appealing strategy is to verify whether well-known epidemiologic characteristics of human malaria are mirrored by AGA malaria parasites. For example, a key feature of human malaria in hyperendemic areas is that infants are faced with both a higher prevalence of parasitaemia and a higher risk of mortality than adults (especially when it comes to malignant malaria) (Doolan *et al.*, 2009). Using non-invasive samples acquired from a wild chimpanzee community, we already showed that the probability of detection of malaria parasites decreases with age (see chapter 2). This suggests that heavily exposed chimpanzees progressively become resistant to infection and/or high parasitaemia, which mirrors observations made on humans.

In humans, another group at risk are pregnant women (e.g., Takem and D'Alessandro, (2013)). They are more vulnerable to both malaria infection and clinical malaria than non-pregnant women, particularly during the first pregnancies (Brabin, 1983; Leke *et al.*, 2010). The main adverse outcomes resulting from malaria in pregnancy in endemic areas are maternal anemia (possibly resulting in maternal mortality), low birth weight due to intra-uterine growth retardation and/or pre-term birth, and possibly miscarriages (Desai *et al.*, 2007). Infants born from women with malaria also seem to face an increased susceptibility to malaria infection during infancy (Bardají *et al.*, 2011). Malaria in pregnancy thus contributes significantly to pregnant women and child morbidity and mortality in endemic areas (Bardají *et al.*, 2011; Guyatt and Snow, 2001).

Pregnancy being a key determinant for reproductive success and infant survival, investigating the relationship between pregnancy and malaria in wild chimpanzee populations is an important step to improve the understanding of what this parasitic infection represents for these populations' fitness. Here, the results of a mixed longitudinal and cross-sectional study during which the probabilities of malaria parasite infection in wild chimpanzees throughout pregnancy and post-pregnancy were determined, are reported.

3.2 Methods

3.2.1 Sample and gestational data collection

A total of 384 faecal samples collected from wild chimpanzees in the TNP, Côte d'Ivoire, during the course of 40 pregnancies (N = 222) and 36 post-pregnancy periods (N = 162) were tested by qPCR for malaria parasites. Faecal samples were collected between 2001 and 2013 from 27 different females, aged 11 to 52 years at the time of sampling and belonging to three habituated groups of the TCP (N = 16, 6 and 5, respectively). Group sizes varied from 16 to 54 individuals. Samples were collected straight after defecation, stored in liquid nitrogen at latest 12 hours after collection and then preserved at -80°C. Note that, for the same population, we already reported an overall average faecal detection rate of 35% and a clear decrease in detection probability with age (Chapter 2).

All pregnancies included in the study resulted in the birth of a viable newborn except for one stillbirth; all post-pregnancy samples were collected from lactating females up to 718 days after birth to avoid samples collected during unnoticed pregnancies due to miscarriages or stillbirths. To estimate gestational ages, conception dates were calculated by subtracting the average chimpanzee pregnancy duration of 225 days (Wallis, 1997), which was also estimated for the study population (T Deschner, personal communication), from infant birth dates. However, based on oestrus data, the stillbirth was estimated to be a premature birth at approximately 6.5 months and its conception date was estimated according to information on oestrus. To make sure that samples were obtained from pregnant females, only samples collected after the latest possible conception date were used (based on the shortest estimated pregnancy duration of 208 days (Wallis, 1997)). For five births, only the

month was known. For these cases the last day of the birth month was used as birth date to calculate conception date, and samples collected during the birth month were excluded.

3.2.2 Molecular analysis

DNA was extracted from pea-sized faecal samples using the EURxGene MATRIX stool DNA purification kit (Roboklon, Berlin, Germany). Screening for malaria parasites was done using a nested qPCR targeting a 90 bp fragment in the non-coding region of the mtDNA (described in Chapter 2). Subsequently, positive samples were further screened with a semi-nested standard PCR (350 bp) targeting a fragment comprising the 3' end of the cox 1 gene, a short intergenic region and the 5' end of the cyt *b* gene (described in Chapter 2) [4]. Semi-nested PCR products for which no distinct sequences were obtained were cloned using a Topo TA cloning kit (Invitrogen™, Karlsruhe, Germany). Following colony PCR, positive colony PCR products were purified and sequenced according to the Sanger's method. Samples were considered positive in either of the following cases: a) *Plasmodium* sequences were obtained directly from semi-nested PCR products (N = 19) or from clones of semi-nested PCR products (N = 1); b) samples positive for the nested qPCR (90 bp) but for which no sequences were obtained (N = 19), i.e. negative for the semi-nested 350 bp PCR or subsequent sequencing and cloning, retested positive by nested standard PCR using nested qPCR primers. These positive samples (N = 8) were then tested for monkey species DNA to avoid false positives resulting from contamination of faeces with *Hepatocystis* sp. after prey (monkey) consumption and were only considered positive if negative for monkey DNA (N = 7); c) *Hepatocystis* sp. sequences were obtained (N = 5) and were negative (N = 1) when tested for monkey DNA.

To test for monkey DNA, a pan-mammal assay targeting a 130 bp fragment of the mitochondrial 16S gene (16Smam1 5 -CGGTTGGGGTGACCTCGGA-3' and 16Smam2 5 -GCTGTTATCCCTAGGGTAACT-3') was used with blocking primers for human and chimpanzee DNA (16Smam_blkhum3 5'-CGGTTGGGGCGACCTCGGAGCAGAACCC--spacerC3 and 16Smam_blksus1 5'-CGGTTGGGGTGACCTCGGAGTACAAAAAAC--spacerC3) (Calvignac Spencer *et al.* (2013) and references therein). Five µl of DNA extract was mixed with 0.5 µl of each primer, 2.5 µl of each blocking primer, 2 µl dNTP (with dUTP replacing dTTP), 0.3 U AmpErase® uracil Nglycosylase (Invitrogen, Carlsbad, CA, USA), 2 mM MgCl2, 1X PCR buffer,

0.25 µl Platinum® Taq polymerase (Invitrogen) and assays were run under the following conditions: 7 min at 45°C, 10 min at 95°C, 42 cycles [30 s at 95°C, 30 s at 64°C, 60 s at 72°C], 10 min at 72°C. This was followed by sequencing of positive samples according to Sanger's methodology. Amplicons for which sequences were not clear were cloned using a Topo TA cloning kit.

The nested standard PCR targeting the same 90 bp fragment as the nested qPCR was performed according to the same protocol, described in Chapter 2, except for the second round which was run using Platinum® Taq (Invitrogen) according to manufacturer's instructions and under the following conditions: 5 min at 95°C, 40 cycles [30s at 95°C, 30 s at 57°C, 45 s at 72°C], 7 min at 72°C.

Sequences were compared with publically available sequences using BLAST (Altschul *et al.*, 1990). All malaria parasite sequences identified in this study exhibited 98-100% sequence identity to published sequences. Sequences were deposited in EMBL under accession numbers: LK995432-LK995453.

Content in mammal mtDNA from all samples was measured as described in Chapter 2.

3.2.3 Phylogenetic tree

To confirm species identification, a phylogenetic tree was built using a dataset comprising the sequences generated for this study (N = 22) and previously reported sequences from haemosporidian parasite species (N = 59) (**Table 3.1**), including all *Plasmodium* species found in AGA. For this analysis, we used the 5' end of the cyt *b* gene that was covered by our confirmatory PCR. One hundred and thirty six sequences obtained from AGA ape faecal samples by Liu *et al.* (2010) (mtDNA-3.4 kilobase pair, accession numbers HM235269–HM235404) were reduced to 25 haplotypes using Fabox v1.4.1 (VILLESEN, 2007); the 34 sequences used in previous analyses by Blanquart and Gascuel (2011) were also included in our dataset. Sequences were aligned using Muscle (Edgar, 2004) as implemented in Seaview v4 (Gouy *et al.*, 2010). Models of nucleotide substitution were compared in a maximum likelihood framework; the Akaike information criterion (AIC) was used to select the model that offered the best fit/complexity trade-off (GTR+G). The maximum likelihood tree was then searched for using PhyML v3 (Guindon *et al.*, 2010) using

the selected model of nucleotide substitution and the BEST tree search algorithm. Branch robustness was estimated through non-parametric bootstrapping (500 pseudo-replicates).

Species names were assigned following the provisional taxonomy proposed in Rayner *et al.* (2011).

Table 3.1. Sequences used for phylogenetic tree

Ha.: *Haemoproteus*, *Pa.*: *Parahaemoproteus*, *L.*: *Leucocytozoon*

Species/strain	Accession No.	Reference
P. falciparum	AY282930	(Joy *et al.*, 2003)
P. gaboni	FJ895307	(Ollomo *et al.*, 2009)
P. reichenowi	AJ251941	(Conway *et al.*, 2000)
P. coatneyi	AB354575	(Hayakawa *et al.*, 2008)
P. cynomolgi	AB434919	(Hayakawa *et al.*, 2008)
P. fieldi	AB354574	(Hayakawa *et al.*, 2008)
P. fragile	AY722799	(Jongwutiwes *et al.*, 2005)
P. hylobati	AB354573	(Hayakawa *et al.*, 2008)
P. inui	AB354572	(Hayakawa *et al.*, 2008)
P. knowlesi	AY722797	(Jongwutiwes *et al.*, 2005)
P. simiovale	AY800109	(Mu *et al.*, 2005)
P. simium	AY722798	(Jongwutiwes *et al.*, 2005)
P. vivax	NC_007243	(Jongwutiwes *et al.*, 2005)
P. sP. DAJ-2004	AY800112	(Mu *et al.*, 2005)
P. gonderi	AY800111	(Mu *et al.*, 2005)
P. malariae	AB354570	(Hayakawa *et al.*, 2008)
P. ovale	AB354571	(Hayakawa *et al.*, 2008)
P. berghei	AF014115	(Tan *et al.*, 2000)
P. chabaudi	AF014116	(Tan *et al.*, 2000)
P. yoelii	M29000	(Vaidya *et al.*, 1989)
P. floridense	NC_009961	(Perkins, 2006)
P. mexicanum	AB375765	(Hayakawa *et al.*, 2008)
P. gallinaceum	AB250690	(Omori *et al.*, 2007)
P. juxtanucleare	AB250415	(Omori *et al.*, 2007)
P. relictum	AY733090	(Beadell and Fleischer, 2005)
Ha. Columbae	FJ168562	(Perkins, 2008)
Pa. jb2.SEW5141	AY733087	(Beadell and Fleischer, 2005)
Pa. jb1.JA27	AY733086	(Beadell and Fleischer, 2005)
Pa. Vireonis	FJ168561	(Perkins, 2008)
L. caulleryi	AB302215	(Omori *et al.*, 2007)
L. fringillinarum	FJ168564	(Perkins, 2008)
L. majoris	FJ168563	(Perkins, 2008)
L. sabrazesi	AB299369	(Hirakawa *et al.*, 2007)
Hepatocystis sp.	FJ168565	(Perkins, 2008)
P._sp._G3-NDgor3203_SGA5.11	HM235382	(Liu *et al.*, 2010)
P._sp._G2-NKgor736_SGA5.6	HM235285	(Liu *et al.*, 2010)
P._sp._G2-NDgor3203_SGA5.4	HM235381	(Liu *et al.*, 2010)
P._sp._G2-NDgor3120_SGA5.5	HM235385	(Liu *et al.*, 2010)
P._sp._G2-LBgor185_SGA2.9	HM235282	(Liu *et al.*, 2010)
P._sp._G1-NKgor736_SGA5.9	HM235286	(Liu *et al.*, 2010)
P._sp._G1-GTgor34_SGA5.23	HM235308	(Liu *et al.*, 2010)
P._sp._G1-DSgor86_SGA5.1	HM235292	(Liu *et al.*, 2010)
P._sp._gorilla-DSgor86_SGA5.6	HM235311	(Liu *et al.*, 2010)

Strain	Accession No.	Reference
*P.*_sp._C3-ONpts1321_SGA5.1	HM235387	(Liu *et al.*, 2010)
*P.*_sp._C3-MBptt189_SGA5.10	HM235342	(Liu *et al.*, 2010)
*P.*_sp._C3-GTptt722_SGA20.3	HM235325	(Liu *et al.*, 2010)
*P.*_sp._C3-BFpts1171_SGA5.7	HM235392	(Liu *et al.*, 2010)
*P.*_sp._C2-YBptt14_SGA5.12	HM235310	(Liu *et al.*, 2010)
*P.*_sp._C2-MTptt157_SGA5.1	HM235332	(Liu *et al.*, 2010)
*P.*_sp._C2-LUpts2078_SGA5.2	HM235320	(Liu *et al.*, 2010)
*P.*_sp._C2-LUpts2074_SGA2.13	HM235400	(Liu *et al.*, 2010)
*P.*_sp._C2-LBptt208_SGA5.11	HM235349	(Liu *et al.*, 2010)
*P.*_sp._C2-LBptt208_SGA5.7	HM235348	(Liu *et al.*, 2010)
*P.*_sp._C2-LBptt208_SGA5.4	HM235346	(Liu *et al.*, 2010)
*P.*_sp._C2-KApts1680_SGA30.11	HM235404	(Liu *et al.*, 2010)
*P.*_sp._C2-GTptt604_SGA5.12	HM235337	(Liu *et al.*, 2010)
*P.*_sp._C1-WEpte440_SGA5.11	HM235364	(Liu *et al.*, 2010)
*P.*_sp._C1-KApts382_SGA5.3	HM235402	(Liu *et al.*, 2010)
*P.*_sp._chimpanzee-LBptt208_SGA5.2	HM235345	(Liu *et al.*, 2010)
*Hepatocystis*_F17.42_	LK995453	This study
*P.*_sp._F17.70_	LK995441	This study
*P.*_sp._F22.38_	LK995442	This study
*P.*_sp._F32.19	LK995439	This study
*P.*_sp._F35.2_	LK995446	This study
*P.*_sp._F35.3__	LK995445	This study
*P.*_sp._F74.19_	LK995434	This study
*P.*_sp._F74.20__	LK995435	This study
*P.*_sp._F74.25b	LK995437	This study
*P.*_sp._F74.27__	LK995438	This study
*P.*_sp._F74.34_	LK995436	This study
*P.*_sp._F74.46_	LK995432	This study
*P.*_sp._F74.49_	LK995433	This study
*P.*_sp._F77.44__	LK995440	This study
*P.*_sp._F293.10_	LK995451	This study
*P.*_sp._F331.14-5_	LK995447	This study
*P.*_sp._F331.14-8_	LK995448	This study
*P.*_sp._F331.20_	LK995449	This study
*P.*_sp._F331.21_	LK995450	This study
*P.*_sp._F682.6_	LK995443	This study
*P.*_sp._F682.8_	LK995444	This study
*P.*_sp._F685.1_	LK995452	This study

3.2.4 Statistical analysis

3.2.4.1 Generalized Linear Mixed Model

To test what influenced the probability of a malaria parasite infection, a GLMM was run (Baayen, 2008). Into this model female status (pregnant *vs* non-pregnant), the number of days since conception or birth, and the age of the female were included as fixed effects. To account for the possibility that the impact of the number of days since conception or birth depends on whether the female is pregnant or not, the interaction between number of days since conception or birth and female status were also included as a fixed effect into the model. Finally, group size and season were included as fixed effects and infant identification, mother identification and group as random effects to control for their potential effects. Season was included as the sine and cosine of the day (counted as number of days elapsed since Jan. 1st 1970) divided by 365.25 (to account for leap years). The ID of the mother, that of the infant (nested within mother ID) and also group were included as random intercepts terms. In order to keep type I error rate at the nominal level of 5% (Barr *et al.*, 2013; Schielzeth and Forstmeier, 2009) random slopes of pregnancy and mother age within mother as well as number of days since conception or birth within infant were included. This is not the maximal model with regard to the random slopes possible (Barr *et al.*, 2013). However, more random slopes terms were not possible to be included since otherwise the model did not converge anymore. Furthermore, among the theoretically possible random slopes, those included are biologically the most reasonable ones. In principle, one would need to control for potential autocorrelation in the response (more precisely in the residuals). However, due to the scarcity of positive samples (28 out of 385) and the facts that for some of the females there were only a few samples as well as some of them being never determined positive this was not possible.

Prior to running the model, the number of days since conception or birth were squareroot transformed (to achieve a roughly symmetrical distribution) and then number of days since conception or birth, mother age and group size were z-transformed to a mean of zero and a standard deviation of one. To control for the quantity of mammal DNA in the sample we included it (log-transformed) as an offset term into the model. The model was implemented in R (R Development Core Team, 2013) using the function glmer of the R

package lme4 (Bates *et al.*, 2013). P-values for the individual effects were determined using likelihood ratio tests (R-function drop1) (Barr *et al.*, 2013). The total sample size for this analysis was 384 data points (from 41 children of 27 mothers out of three groups).

To test for the overall effect of the test predictors (females status, number of days since conception or birth, their interaction and female age) we compared the full model (Forstmeier and Schielzeth, 2011) as described above with a null model lacking these terms but comprising all other terms present in the full model using a likelihood ratio test (Dobson, 2002). With regard to the assumptions, collinearity was no obvious issue (maximum Variance Inflation Factor determined from a standard linear model lacking the random effects: 1.31; (Field, 2005; Quinn and Keough, 2002). We assessed model stability by excluding the levels of the different random effects one at a time, running the model for the derived reduced data sets, and comparing the estimated coefficients with those we got for all data. This revealed the model to be satisfactory stable (**Table 3.2**).

The same model was also run using only the samples for which sequences were obtained (N = 21) as positive response for malaria parasite infection.

Table 3.2. Range of estimates obtained from running the GLMM after excluding the levels of the different random effects one at a time

Numbers represent minimum and maximum values of coefficient estimates, s.e., z and p-values, obtained after excluding levels of the random effects one at a time

Minima	estimate	s.e.	z	p	**Maxima**	estimate	s.e.	z	p
intercept	-6.516	0.652	-6.729	<0.001	intercept	-4.385	1.183	-5.102	<0.001
pregnant	0.728	0.710	1.026	0.026	pregnant	2.137	1.212	2.230	0.305
z.infant.age	-0.718	0.456	-0.927	0.354	z.infant.age	-0.132	1.025	-0.219	0.827
z.mother.age	**-1.374**	**0.398**	**-2.641**	**0.008**	**z.mother.age**	**-0.847**	**0.570**	**-1.857**	**0.063**
z.group.size	-0.298	0.375	-0.684	0.494	z.group.size	0.280	0.739	0.500	0.988
sin(season)	-0.551	0.455	-1.135	0.256	sin(season)	0.159	0.661	0.316	0.886
cos(season)	0.098	0.469	0.180	0.299	cos(season)	0.542	0.697	1.039	0.857
pregnant:z.infant.age	**1.857**	**0.928**	**1.900**	**0.005**	**pregnant:z.infant.age**	**3.86**	**1.727**	**2.779**	**0.057**

3.2.4.2 Cox proportional hazards model

What influenced infant survival was also tested for, by running a Cox proportional hazards mixed model (Therneau, 2012). Into this, mother malaria infection during pregnancy

(positive vs. negative) and mother age at infant birth (to control for) were included as fixed effects. As random intercepts terms, the ID of the mother and also group were included. We are aware that this is not the maximal model with regard to the random slopes possible (Barr *et al.*, 2013); however, random slopes terms could not be included due to insufficient data (lack of variation of age and positivity within mother). To account for the sampling effort (number of samples per female and pregnancy) the model was weighted by this variable.

To test for the overall effect of the two fixed effects (mother positivity to malaria infection during pregnancy and mother age) we compared the full model as described above with a null model lacking these terms (Forstmeier and Schielzeth, 2011) but comprising the same random effects as the full model using a likelihood ratio test (Dobson, 2002). We implemented the model in R (version 3.0.2; R Core Team 2013) using the function coxme of the R package coxme (Therneau, 2012). The total sample size for this analysis was 41 infants of 27 mothers out of 3 groups. By the end of the study period (i.e. 2001 - 2013), 59% of the infants (N = 24) had died; and 83% (N = 20) of these had died before or at the age of five years.

We assessed model stability by excluding the levels of the random effect of mother ID one at a time, running the model for the derived reduced data sets, and comparing the estimated coefficients with those we got for all data.

3.3 Results

3.3.1 Molecular analysis

Overall, 7.3% (N = 28) of the samples were positive for malaria parasites (**Figure 3.1**). This included 9% of the samples collected during pregnancy, and 4.9% of the samples collected during post-pregnancy. Of all females investigated, 48% were positive at least once. Sequences were obtained for 75% of the positive samples (N = 21). Malaria species sequenced included *P. gaboni* (N = 8), *P. reichenowi* (N = 5), *P. billcollinsi* (N = 5), *P. vivax* (N = 1), *Hepatocystis* sp. (N = 1), and one mixed infection (*P. gaboni* and *P. reichenowi*) (**Figure 3.2**).

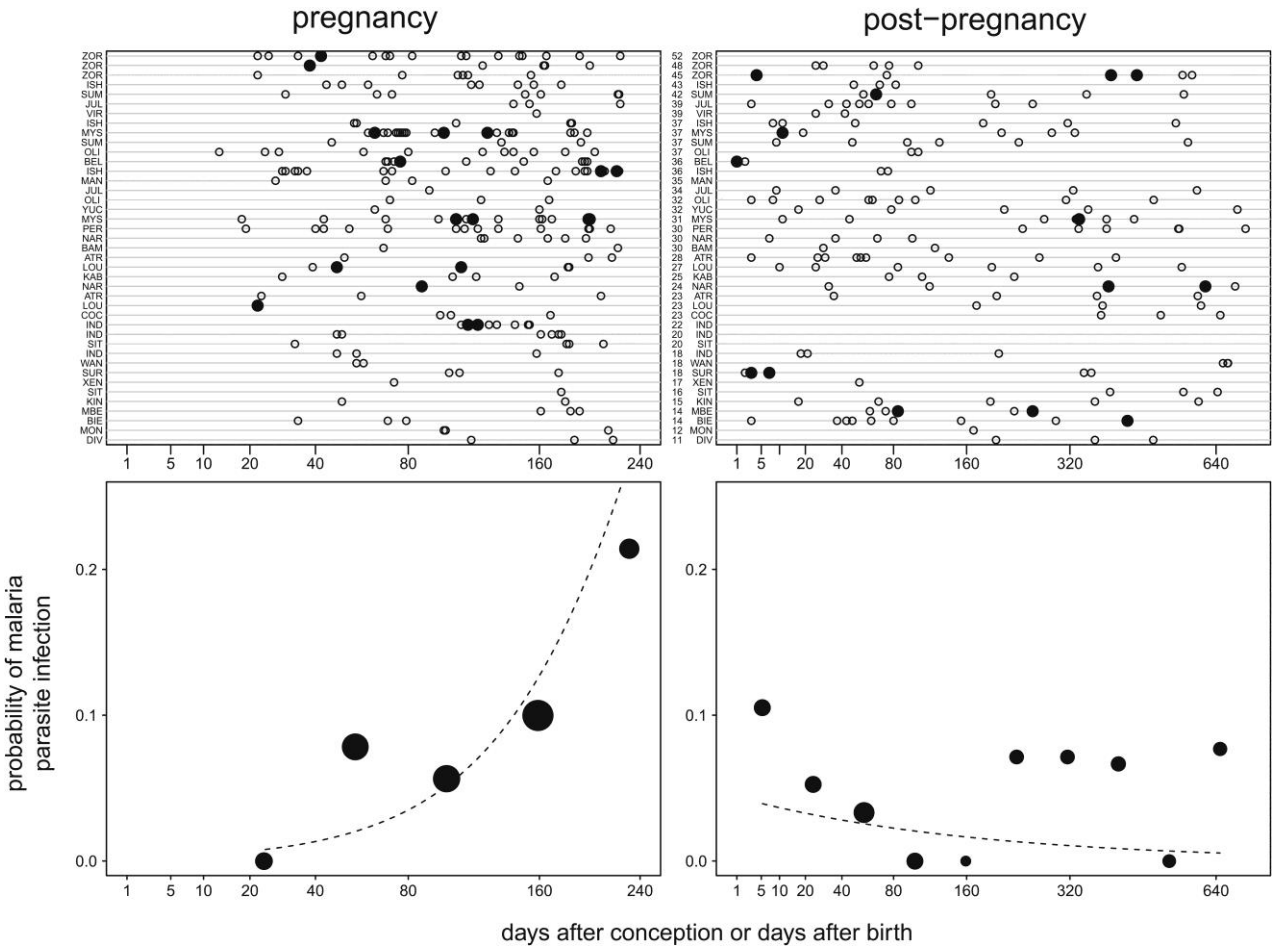

Figure 3.1. Probability of malaria parasite infection as a function of time during and after pregnancy

The upper panel shows data points per pregnancy and/or post-pregnancy over time (filled dots: malaria positive, open dots: malaria negative). Pregnancies and post pregnancies are referred to by the mother's name (three-letter abbreviation) and ranked by increasing age of the mother at infant birth. The lower panel shows the probability of malaria parasite infection as a function of gestational age (bottom left) or age of the infant after birth (bottom right; age in days). The points depict the proportion of samples tested positive, the area of the points corresponds to the number of samples tested in the respective age class and the dashed lines show the probability of malaria positivity per 200,000 units mammal_mt_DNA.

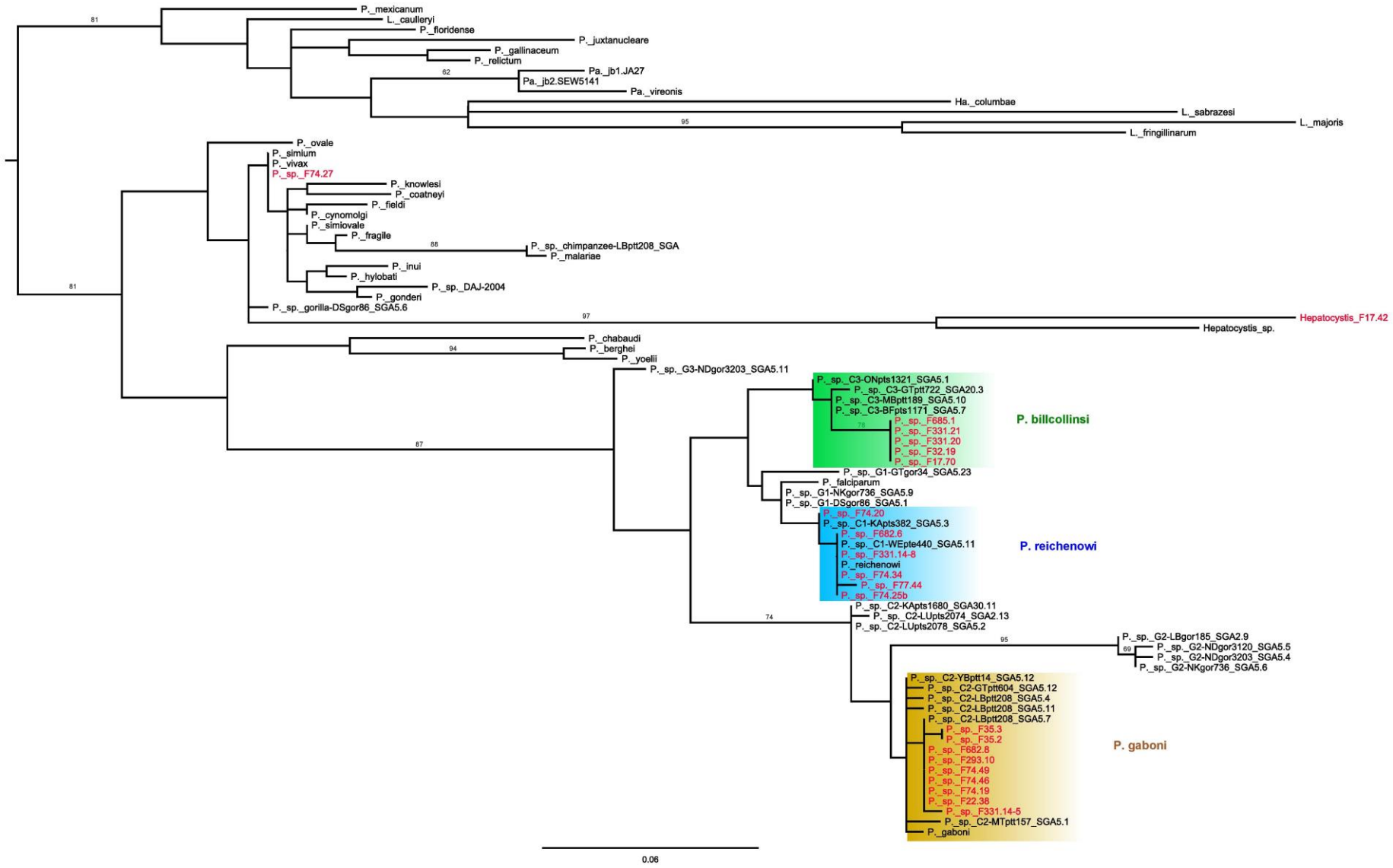

Figure 3.2. Phylogenetic tree of a partial cytochrome b gene fragment from 81 sequences of haemosporidian parasites

Bootstrap values are given above branches. This tree was mid-point rooted. Scale is in substitution per site.

3.3.2 Statistical model

3.3.2.1 Generalized Linear Mixed Model

The full model was clearly significant as compared to the null model (likelihood ratio test: $\chi 2 = 18.92$, d.f. = 4, p = 0.001). During pregnancy the probability of infection increased with the infant's age (gestational age), but not obviously so after the infant's birth (test of the interaction between days after birth or conception and mother pregnancy status: estimate + s.e. = 2.47 + 1.02, z = 2.42, p = 0.016; **Table 3.3** and **Figure 3.1**). Moreover, the probability of infection decreased with mother's age (estimate+ s.e. = -1.00 + 0.43, z = -2.32, p=0.02; **Figure 3.3**). Similar results were obtained with a more conservative set of positives (likelihood ratio test: $\chi 2 = 13.44$, d.f. = 4, p = 0.009; interaction between days after birth or conception and mother pregnancy status: estimate + s.e. = 2.22 + 1.17, z = 1.91, p = 0.056; mother's age: estimate + s.e. = -1.15 + 0.46, z =-2.48, p = 0.013). It is important to emphasize that by controlling for sample content in mammal DNA, it was ensured that none of these observations was driven by an increased quantity of (parasite-containing) blood or other body fluids in the faeces.

Table 3.3. Results of GLMM model with mammal_mt_DNA as the offset term

Number of days since conception or birth (child age) were squareroot transformed and then z-transformed, mother age and group size were z-transformed.

term	estimate	s.e.	Z	p
intercept	-5.023	0.793	-6.334	0.000
pregnant	1.548	0.819	1.891	0.059
child age	-0.461	0.628	-0.734	0.463
mother age	-0.996	0.430	-2.318	0.020
group size	-0.127	0.411	-0.310	0.756
sin(season)	-0.323	0.484	-0.667	0.505
cos(season)	0.298	0.506	0.588	0.556
pregnant : child age	2.474	1.023	2.419	0.016

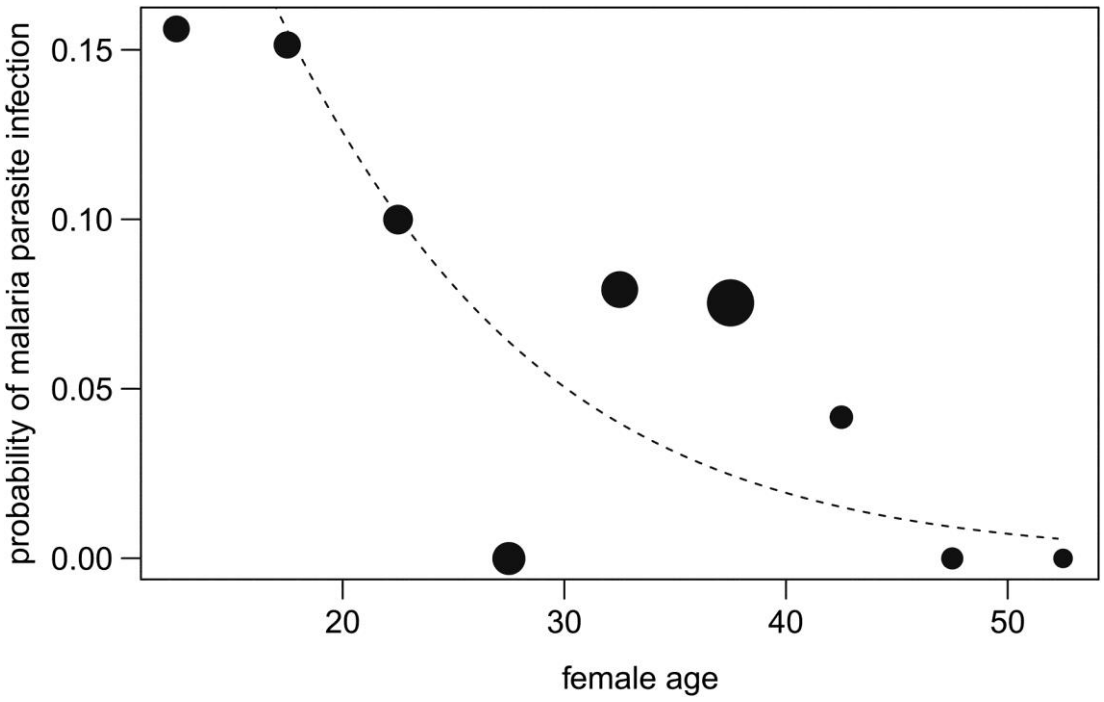

Figure 3.3. Probability of malaria parasite infection as a function of female age

The area of the points corresponds to the number of samples in the respective age class and the dashed line indicates the probability of malaria positivity per 200,000 units mammal_mt_DNA.

3.3.2.2 Cox proportional hazards model

Overall, the full model was clearly significant as compared to the null model (likelihood ratio test: $\chi 2 = 34.16$, d.f. = 2, $p < 0.001$). We found a significant effect of mother age (estimate + s.e. = 0.19 + 0.04, $z = 4.61$, $p < 0.001$), which was relatively stable (range of coefficients obtained from model stability checks: 0.082 to 0.964). This suggests that infant survival decreased with increasing age of the mother at infant birth. However, this needs to be interpreted with caution as there was some degree of uncertainty, sample size was limited and the model might have been anti-conservative due to the lack of random slopes.

The effect malaria infection of the mother during pregnancy only showed a trend towards reduced infant survival when the mother was positive during pregnancy (estimate + s.e. = 0.52 + 0.30, $z = 1.70$, $p = 0.089$), with a large degree of uncertainty (range of

coefficients obtained from model stability checks: -0.38 to 4.59). The effect of positivity during pregnancy thus remained inconclusive, and a larger data set would be required to further investigate this question.

3.4 Discussion

Epidemiological studies in human populations have shown that prevalence of *Plasmodium* spp. infection (peripheral parasitaemia detected by *Plasmodium* spp. slide positivity (Brabin, 1983; Bray and Anderson, 1979; McGregor, 1984) or by PCR (Taylor *et al.*, 2011)) and parasite density (Brabin, 1983; Bray and Anderson, 1979; McGregor, 1984) are higher during pregnancy. Similarly, the results of the present study show that the probability of malaria parasite detection in adult female chimpanzees also increases during pregnancy. This suggests that, as in humans, pregnant female chimpanzees are more susceptible to infection or more likely to present higher parasitaemia (note that the latter explanation is favored to explain increased detection among pregnant women (Leke *et al.*, 2010)). Of note, the peak of detection seems to occur at a different gestational age than in humans, e.g. towards the end of pregnancy in chimpanzees in contrast to the second (Brabin, 1983) or first two (Bray and Anderson, 1979) trimesters in humans.

There are two main explanations to the exacerbated susceptibility to malaria parasites seen in pregnant women: immune alterations and the existence of placenta-tropic malaria parasites (Takem and D'Alessandro, 2013). Pregnancy is obviously associated with important hormonal changes which impact on the immune system. Secretion of cortisol, a well-known immunosuppressive hormone, continuously increases during pregnancy. Malaria infection and parasite load are positively related to cortisol levels in primigravid women (Bouyou-Akotet *et al.*, 2005), suggesting that an increased cortisol level effectively leads to decreased resistance to *Plasmodium*. This might be explained by this hormone's action on cell-mediated immunity, which, unlike humoral immunity, appears to be generally depressed during pregnancy (Luppi, 2003). *De facto*, cell mediated immune response towards malaria antigens is down-regulated during pregnancy (Fievet *et al.*, 1995; Riley *et al.*, 1989). The temporary impairment of the immune function (possibly mediated by cortisol and other pregnancy-associated hormones) might thus contribute to the increased susceptibility of

pregnant women. Interestingly, pregnant chimpanzees also experience increased cortisol levels during gestation (Smith *et al.*, 1999). Cortisol reaches a peak concentration during late pregnancy (Smith *et al.*, 1999), which is coincident with the increased probability of malaria parasite detection towards the end of pregnancy reported in this study. It is likely that cortisol increases result in immune impairment although this would obviously need further investigations. It is noted here that the non-invasive assessment of the immune function of wild chimpanzees, although being the only ethical option (F H Leendertz *et al.*, 2006), will be extremely challenging.

In humans, the prevalence of malaria in pregnancy has been observed to decrease with increasing parity in women living in endemic areas (and therefore otherwise malaria-immune), which has led to the identification of a subset of *P. falciparum* parasites that express high levels of a variant surface antigen (*var*2CSA) allowing them to selectively target placenta (Salanti *et al.*, 2004). Pregnant (particularly pauciparous) women might, therefore, face an enhanced susceptibility because they are naïve to this subpopulation of placenta-tropic parasites, against which they will only mount an effective immunity after several pregnancies. The relationship between peripheral blood parasitaemia and placental parasitaemia is not clearly established (they can occur simultaneously or not) (Brabin *et al.*, 2004) and exploring the occurrence of placental malaria in chimpanzees through placental blood analysis or histopathology will most likely reveal unfeasible. However, homologues of the *var*2CSA gene have been found in the genomes of malaria parasite species infecting chimpanzees, including *P. reichenowi* and *P. gaboni* (Pacheco *et al.*, 2013; Trimnell *et al.*, 2006). As *var* gene repertoires fluctuate considerably, including within malaria parasite species, e.g. only three of the ~60 *var* genes are found in all *P. falciparum* genomes (Rask *et al.*, 2010), such a degree of conservation across great ape *Plasmodium* spp. may indicate selection-driven maintaining (Pacheco *et al.*, 2013). The observations reported here are compatible with (but no proof of) the notion that the adaptive advantage conferred by *var*2CSA to Laveranian parasites may have consisted in an increased access to pregnant females.

The decline in malaria parasite detection with increasing age of the females, also observed in humans (Bouyou-Akotet *et al.*, 2003; Walker-Abbey *et al.*, 2005), confirms the influence of age on malaria susceptibility in chimpanzees (Chapter 2). The effect of maternal

age might be combined with an effect of increasing gravidity on resistance to malaria parasites, similar to what is seen in humans (Bouyou-Akotet *et al.*, 2003; Walker-Abbey *et al.*, 2005). It is however difficult to distinguish one effect from the other due to the small number of positives in this study and the fact that, as an inherent consequence of the chimpanzees' natural history, age and gravidity are strongly inter-related.

Knowing that pregnant female chimpanzees are less resistant against malaria parasite infection raises the question of the impact of the infection on pregnancy outcome, infant survival and, thus, general reproductive success of the chimpanzee community. So far, 49% of the infants included in this study died at an age below or equal to five years (nine of the currently living infants have not yet reach the age of five years) which is close to what was found before (44% according to Boesch and Boesch-Achermann (2000)), and 24% died before reaching an age of one year. Three of the latter died of known causes other than malaria. Causes were unknown for six, and one was a stillbirth with malaria infection of the mother during the second trimester. If infections with AGA malaria parasites during chimpanzee pregnancy had consequences similar to malaria in pregnancy in humans - premature birth, low birth weight, enhanced risk of contracting malaria during infancy (Bardají *et al.*, 2011; Desai *et al.*, 2007) - it could be responsible for part of these mortalities. A model was run to test the effect of malaria infection of the mother during pregnancy on infant survival, which revealed a trend towards reduced survival, but results remained inconclusive due to the limited sample size and the instability of the model. Gathering more data on malaria parasite infection occurrence during chimpanzee pregnancy and on infant morbidity and mortality is clearly needed before reaching more definite conclusions. Thus far, a larger dataset could not be assembled, although the chimpanzee community living in the TNP has been closely monitored for more than 30 years and non-invasive samples have been collected for more than 15 years.

3.5 Conclusion

Together with the study on the effect of age on malaria infection (see chapter 2), this study further indicates a similarity between the dynamics of malaria infection in chimpanzee and human populations from endemic areas, with young age and pregnancy constituting

intrinsic risk factors for malaria infection. As anything affecting pregnancy ultimately affects reproductive success and infant survival, and therefore population health, whether malaria infection is, as in humans, associated with disease will be an important avenue of investigation.

CHAPTER 4

A CAUTIONARY NOTE ON FAECAL SAMPLING AND MOLECULAR EPIDEMIOLOGY IN PREDATORY WILD GREAT APES

Based on the manuscript: De Nys HM, Madinda NF, Merkel K, Robbins M, Boesch C, Leendertz FH and Calvignac-Spencer S. 2015. A cautionary note on fecal sampling and molecular epidemiology in predatory wild great apes. American Journal of Primatology. doi: 10.1002/ajp.22418

4.1 Introduction

An increasing number of studies rely on non-invasively collected samples to investigate microorganisms infecting great apes and other primates (here and in the following microorganism designates parasitic microorganisms, whether viral, prokaryotic or eukaryotic). This is often done by searching for nucleic acid fragments of microrganisms in faecal samples. The detection and/or sequencing of such fragments can allow for the discovery of novel microorganisms, the characterization of their genetic diversity or the assessment of their prevalence, *inter alia*. Nucleic acid detection is most often taken as indicative of host infection (**Table 4.1**).

However, an issue sometimes discussed but seldom controlled for is that true parasitism actually remains uncertain if the host is carnivorous (Blinkova *et al.*, 2010; De Nys *et al.*, 2014; Duval *et al.*, 2010). Microorganism nucleic acids found in faeces could as well originate from microorganisms infecting prey and thus reflect the diet (ingestion and excretion) rather than infection status of the host. For instance, in the study on malaria parasite infection in wild chimpanzees reported in chapter 3, we found that 20% of the faecal samples from which malaria parasite sequences were obtained actually contained *Hepatocystis* sp. sequences. *Hepatocystis* sp. are malaria parasites known to infect monkeys and bats (Ayouba *et al.*, 2012; Schaer *et al.*, 2013), and which, to our knowledge, have never been identified from great ape tissues. This pointed at the possible dietary origin and presence of monkey material in the faeces, which we could test for. However, when knowledge about microorganisms is scarce, disentangling the effects of diet and actual infection may be tricky. For example, Blinkova *et al.* (2010) and Duval *et al.* (2010) discussed the possibility that some of the novel circular ssDNA viruses they identified from chimpanzee faeces could be of plant or animal origin. Moreover, microorganism prevalence as estimated through faecal detection rates (i.e. proportions of faecal samples which test positive) tends to be relatively low in chimpanzees, mostly falling <20% among 30 studies performed between 2003 and 2014 (**Figure 4.1 and Table 4.1**). Many studies are thus likely to be significantly impacted by even low-frequency contamination with genetic material of microorganisms infecting prey. Also, chimpanzees sometimes prey on other NHP species

(Boesch and Boesch-Achermann, 2000). Due to the close evolutionary relationships between primate prey and chimpanzees, primate prey may host microorganisms closely related to those infecting chimpanzees, and lead to a misinterpretation of the results.

How unaccounted-for "contamination" of faeces with the DNA of microorganisms infecting the prey will influence the interpretation of faecal epidemiology in predatory great apes depends on hunting frequency and microorganism nucleic acid persistence. Measuring the frequency of hunting through direct observations is possible but only where great apes have been habituated (Boesch and Boesch-Achermann, 2000); and to our knowledge, microorganism nucleic acid persistence through the digestive tract of great apes has never been assessed (whatever the parasite). By means of molecular tools, it is possible to screen faecal samples for prey DNA (Symondson, 2002), as previously done to study the diet of great apes (Hofreiter *et al.*, 2010) and many other predators (O'Rorke *et al.*, 2012). Here, we use molecular methods to detect prey DNA in faecal samplings of two distinct chimpanzee communities, using faecal samples of western lowland gorillas as negative control (as they are not carnivorous), to assess to what extent this is prevalent ("prey prevalence") and could effectively pose a risk of false interpretation in faecal epidemiology studies. We propose that molecular detection of prey prevalence be used as a control for diet-induced microorganism detection in carnivorous great apes. This will simultaneously provide information on hunting frequency (directly and even where apes are not habituated) and possible persistence of genetic material of microorganisms infecting prey (indirectly, through prey genetic material persistence).

Table 4.1. Examples of prevalence of microorganisms estimated non-invasively between 2003 and 2014

Most prevalence estimates are based on nucleic acid detection and/or antibody detection in faecal samples from wild chimpanzees, and represent the proportion of positive samples or infected individuals. Multiple prevalence rates reported by one same study are estimates for different field sites or chimpanzee communities. [1]Prevalence estimates for a combination of several non-human primate species, [2]combined results from tissue samples and faecal samples, [3]Combined results from faecal and urine samples.

Microorganism	Prevalence (%)	Reference
Adenovirus	40.0	(Roy *et al.*, 2009)
Adenovirus[1]	59.0	(Wevers *et al.*, 2011)
Circular DNA viruses	18.0	(Blinkova *et al.*, 2010)
Circular DNA viruses	14.0	(Li *et al.*, 2010)
Enteroviruses	14.8	(Harvala *et al.*, 2011)
Enteroviruses	13.8	(Harvala *et al.*, 2013)
Enteroviruses	10.4	(Sadeuh-Mba *et al.*, 2014)
Hepatitis B virus	2.2	(Makuwa *et al.*, 2005)
Plasmodium spp.	32.0	(Liu *et al.*, 2010)
Plasmodium spp.	48.0	(Liu *et al.*, 2010)
Plasmodium spp.	34.0	(Liu *et al.*, 2010)
Plasmodium spp.	35.0	(De Nys *et al.*, 2013)
Plasmodium spp.	7.3	(De Nys *et al.*, 2014)
Plasmodium spp.	17.6	(Prugnolle *et al.*, 2010)
Plasmodium vivax	8.0	(Liu *et al.*, 2014)
Plasmodium vivax	4.0	(Liu *et al.*, 2014)
Polyomaviruses[1]	3.0	(Scuda *et al.*, 2013)
Simian foamy virus	45.0	(Liu *et al.*, 2008)
Simian foamy virus	86.5	(Blasse *et al.*, 2013)
Simian immunodeficiency virus	5.9	(Neel *et al.*, 2010)
Simian immunodeficiency virus	32.0	(Rudicell *et al.*, 2011)
Simian immunodeficiency virus	13.4	(Li *et al.*, 2012)
Simian immunodeficiency viurs	4.4	(Keele *et al.*, 2006)
Simian immunodeficiency virus	35.3	(Keele *et al.*, 2006)
Simian immunodeficiency virus[3]	5.0	(M L Santiago *et al.*, 2003b)
Simian immunodeficiency virus[3]	30.0	(M L Santiago *et al.*, 2003b)
Simian immunodeficiency virus	12.1	(Rudicell *et al.*, 2010)
Simian immunodeficiency virus	46.1	(Rudicell *et al.*, 2010)
Simian immunodeficiency virus	13.7	(Keele *et al.*, 2009)
Simian immunodeficiency virus	4.7	(Van Heuverswyn *et al.*, 2007)
Simian immunodeficiency virus	34.5	(Van Heuverswyn *et al.*, 2007)
Helicobacter spp.	74.0	(Flahou *et al.*, 2014)
Rickettsia spp.	9.9	(Keita *et al.*, 2013)
Clostridium perfringens	23.0	(Fujita and Kageyama, 2007)
Clostridium perfringens	1.2	(Fujita and Kageyama, 2007)
TT virus	80.0	(Barnett *et al.*, 2004)
TT virus	100.0	(Barnett *et al.*, 2004)
Lymphocryptovirus[1,2]	50.0	(Ehlers *et al.*, 2010)
Parvovirus bocavirus	7.4	(Sharp *et al.*, 2010)

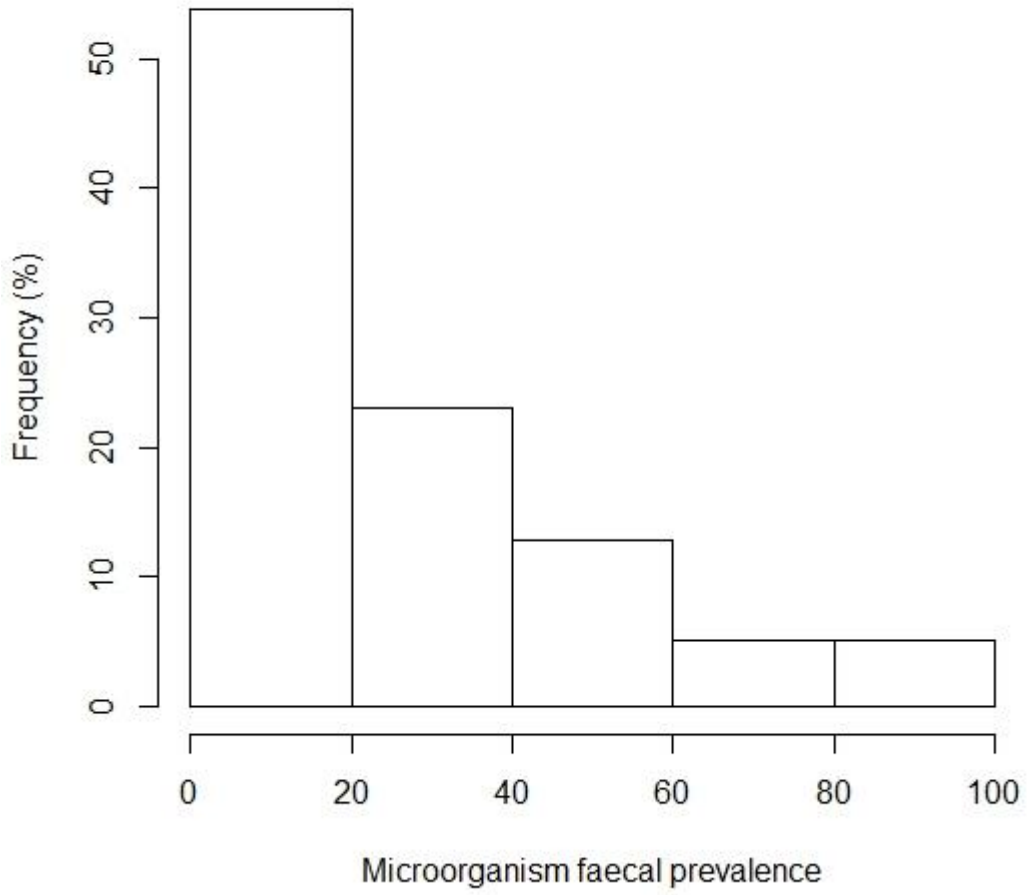

Figure 4.1. Frequency plot of faecal prevalence of microorganisms determined in 30 studies from 2003 to 2014

All studies focused on microorganisms infecting wild chimpanzees. A complete list of these studies and the according prevalence can be found in **Table 4.1**.

4.2 Methods

4.2.1 Study populations and sample collection

One set of faecal samples (N = 107) was collected from one habituated group of wild western chimpanzees (*P. t. verus*, TNP, Côte d'Ivoire; hereafter referred to as Taï) over 41 collection days. Samples were selected to represent all sexes and age classes (7 females and 12 males ranging from 3 to 47 years old), with an average of 5.6 samples per individual, and covered two time periods (October–November, 2011 and March-April, 2012). Taï chimpanzees are known to hunt the whole year round but hunting frequency increases from mid-August to mid-November. They primarily hunt NHP species, with red colobus (*Piliocolobus badius*) being by far their main prey (Boesch and Boesch-Achermann, 2000).

Another set of faecal samples (N = 109) was collected from non-habituated wild central chimpanzees (*P. t. troglodytes*, Loango National Park, Gabon; hereafter referred to as Loango) over 55 collection days. These were collected opportunistically on a daily basis during the habituation process of one chimpanzee community, from either beneath chimpanzee nests or where the chimpanzees had been during the day (uneven sampling of individuals is likely). Loango chimpanzees are also known to hunt but seem to prey on a broader range of vertebrates than Taï chimpanzees, e.g. NHP, duikers, tortoises (NF Madinda, M Robbins and C Boesch personal communication).

Finally, a third set of faecal samples (N = 92) collected in Loango from western lowland gorillas (*G. g. gorilla*) over 37 collection days was used as negative control. Gorillas have never been observed to hunt, nor have remains of prey been observed in their faeces. A first molecular study of gorilla diet, also in Loango, identified mammal DNA sequences in their faeces (Hofreiter *et al.*, 2010). The authors carefully discussed this finding and mentioned the possibility of environmental or laboratory contamination. Assuming gorillas don't prey upon mammals, this third set of samples was used here as a negative control.

Samples collected in Taï were frozen on the day of collection and stored in liquid nitrogen; samples collected in Loango were preserved in RNALater (Qiagen, Hilden, Germany) and stored at room temperature for 1 to 3 months before long-term storage at -20°C.

4.2.2 Molecular analysis

DNA was extracted from faecal samples using a EURx Gene MATRIX stool DNA purification kit (Roboklon, Berlin, Germany).

We first screened all extracts with a PCR assay targeting an approximately 130 bp fragment of the mitochondrial 16S ribosomal RNA gene (16S). This assay was deliberately chosen to be undirected with respect to prey: the primers used for amplification have been shown to amplify a broad range of mammals (all primer sequences are provided in **Table 4.2**; (Taylor, 1996)). As prey DNA is most often largely overcome by predator DNA, we used methods recently developed for carnivore diet analysis (O'Rorke *et al.*, 2012), by adding a blocking primer (Shehzad *et al.*, 2012; Vestheim and Jarman, 2008) to prevent the amplification of great ape DNA (Boessenkool *et al.*, 2012); we also added a pig blocking primer as in our experience this system is sensitive to laboratory contamination with pig DNA (Calvignac-Spencer *et al.*, 2013). PCR assays were performed in a total volume of 25 µL and contained 0.2 micromolar (µM) of each primer, 1 µM of each blocking primer, 200 µM dNTP (with dUTP replacing dTTP), 0.3 U AmpErase® uracil N glycosylase (UNG; Invitrogen, Carlsbad, CA, USA), 4 mM $MgCl_2$, 2,5 µl 10X PCR buffer and 0.25 µl Platinum® Taq polymerase (Invitrogen). Note that UNG was included as a way to clean reactions from contaminating PCR products generated beforehand (any PCR reaction run in the laboratory is run with dUTP). All reactions were seeded with 3 µL DNA extract and were run under the following conditions: 7 min at 45°C (UNG activity), 10 min at 95°C, 42 cycles [30 s at 95°C, 30 s at 64°C, 60 s at 72°C], 10 min at 72°C.

Blocking primers often do not completely abrogate the amplification of host templates (O'Rorke *et al.*, 2012). Prey-positive DNA extracts are these extracts in which (imperfectly) blocked host template amplification during PCR results in shifting the initial prey/host ratio sufficiently. In other DNA extracts prey DNA may be present but the shift induced by blocking may be insufficient to allow for prey DNA to dominate amplicon composition. Estimates derived from blocking primer approaches are necessarily underestimates. The Taï sample set offered a good opportunity to assess the extent of this underestimation/the sensitivity of the first assay. As Taï chimpanzees predominantly hunt colobine monkeys (95% of their prey; (Boesch and Boesch-Achermann, 2000), we tested this sample set with a specific colobine assay targeting a 122 bp fragment of the mitochondrial

12S ribosomal RNA gene (12S); this assay was shown to amplify DNA from all colobine species occurring in Taï (Schubert *et al.*, 2014). All samples that were positive with this assay were then also tested using another specific colobine assay targeting an approximately 480 bp fragment of the mitochondrial control region (CR; (Minhos *et al.*, 2013)) and rarely used in our laboratory, in order to further control for the possibility of contamination by PCR products. PCR mixes were prepared as mentioned above and cycling conditions differed only with respect to number of cycles and annealing temperatures: 47 cycles with annealing at 65°C for the 12S colobine system, 50 cycles with annealing at 63°C for the CR colobine system.

PCR products were sequenced according to Sanger's methodology. Sequences were assigned to species or the lowest possible higher taxon using BLAST results (Altschul *et al.*, 1990) and available biological information (e.g. phylogenetic relatedness and reported presence in the area).

Table 4.2. Primers used in Chapter 4

mt: mitochondrial

Target	Targeted sequence	Approx. fragment length (bp)	Primer name	Sequence (5'-3')	Reference
Mammal	mt 16S DNA	130	16Smam1_f	CGGTTGGGGTGACCTCGGA	(Taylor, 1996)
			16Smam3_r	CTCGATGTTGGATCAGGACATC	(Calvignac-Spencer *et al.*, 2013)
			16Smam_blkhum3	CGGTTGGGGCGACCTCGGAGCAGAACCC—spacerC3	(Boessenkool *et al.*, 2012)
			16Smam_blkpig	CGGTTGGGGTGACCTCGGAGTACAAAAAAC—spacerC3	(Calvignac-Spencer *et al.*, 2013)
Colobinae	mt 12S rRNA	122	Colo_f	CGATTGACCCGAGCTAATAGRY	(Schubert *et al.*, 2014)
			Colo_r	CCACTTTCGTAGTTTATTTTACATTG	(Schubert *et al.*, 2014)
Colobinae	mt CR	480	L15449Clbs	CCRCCAATA CCCAAAACTGG	(Minhos *et al.*, 2013)
			H15973Clb	AGGAGAGTAGCA CTCTTGTGC	(Minhos *et al.*, 2013)

4.3 Results

Using the mammal 16S assay, mtDNA other than host DNA was detected in 5.6% and 3.7% of the Taï chimpanzee and Loango chimpanzee faecal samples, respectively, as well as in 2.2% of the Loango gorilla faecal samples. These sequences were assigned to red colobus

(*Piliocolobus badius*, N = 6) for chimpanzees from Taï; moustached guenon (*Cercopithecus cephus*, N = 3) and bay duiker (*Cephalophus dorsalis*, N = 1) for chimpanzees from Loango; and forest elephant (*Loxodonta cyclotis*, N = 2) for gorillas from Loango.

Using the 12S colobine assay to estimate sensitivity of the first assay, sequences were obtained from 23.4 % (N = 25) of the Taï samples (35.8% for October-November and 11.1% for March-April; **Figure 4.2**). Most were identified as red colobus (N = 20), including the six samples which had tested positive for red colobus in the first assay. Black-and-white colobus (*Colobus polykomos*, N = 4) and olive colobus (*Procolobus verus*, N = 1) were also identified. With the CR colobine assay, sequences were obtained for 18 of the 12S colobine positive samples (72%). The preponderance of red colobus (N = 15) as well as the detection of both black-and-white (N = 2) and olive colobus (N = 1) were confirmed. The CR fragment exhibited considerable variability and this allowed for the identification of 8 red colobus, 1 black-and-white and 1 olive colobus haplotypes (**Figure 4.2**). When a given haplotype was detected in several individuals, positive samples had generally been collected over a very short time frame, for example 1 or 2 days (**Figure 4.2**), suggesting haplotypes could be bound to collective hunting/sharing events.

Under the assumption of a perfect sensitivity of the 12S assays, sensitivity of the mammal 16S assay was estimated at 24% for Taï samples (95% confidence interval (CI): 7%-41%). Based on this sensitivity, we extrapolated that prey prevalence in chimpanzee faeces from Loango was 15.3 % (95% CI: 9%-52.4%).

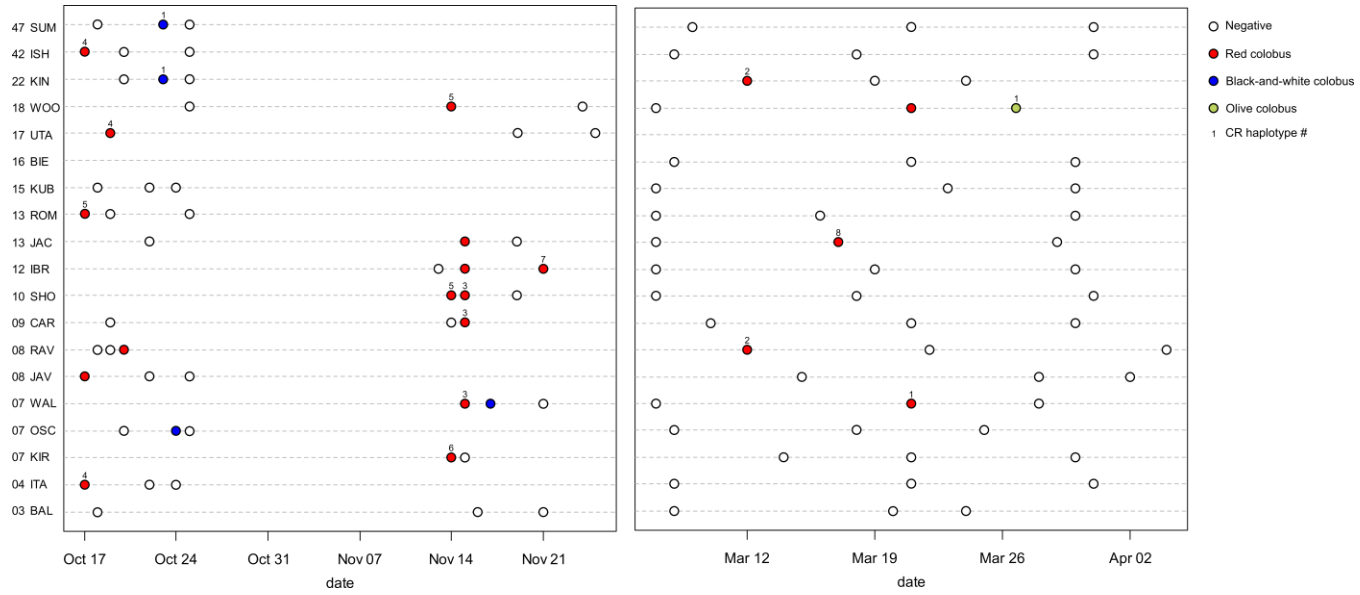

Figure 4.2. Prey detection through time in Taï

Individual chimpanzees are represented by a three letter abbreviation, preceded by their age at time of sampling. Mitochondrial control region haplotypes are identified by numbers above the corresponding samples. Note that the CR fragment could not be amplified from all positive samples (a likely side-effect of it being much larger than for the other colobine-specific system that we used).

4.4 Discussion

Our estimates of prey prevalence, whether measured (Taï) or extrapolated (Loango), are high with >15% of the samples containing or likely to contain prey DNA (15.3% in Loango and 23.4% in Taï), and with seasonal peaks in Taï during which one sample in three contains prey DNA. These values are in the range of the majority of faecal detection rates of microorganisms in studies performed between 2003 and 2014 (**Figure 4.1** and **Table 4.1**). Therefore, our findings certainly have implications for molecular epidemiology based on faecal sampling.

There are two main factors that will determine whether a study is at risk of being biased by unaccounted-for detection of microorganisms infecting prey. As just discussed, the prevalence of the microorganism of interest in faeces is of importance. If it is high, and sensitive molecular detection methods are used, microorganism detection rate will be high

and possibly higher than prey prevalence estimates. In such cases, controlling for prey contamination will only be of interest if obtaining an accurate estimate of microorganism faecal prevalence is of importance. If microorganism faecal prevalence is low, microorganism detection rate will be low (irrespective of the detection method sensitivity) and likely fall in the range of typical prey prevalence. In that case, the main concern will be to determine if microorganism and prey detection are independent. This could be achieved by determining prey detection rates in microorganism-positive and a subset of microorganism-negative samples and ensuring they do not differ significantly (e.g. through a χ^2 test of independence).

Whether such control is necessary to confidently state that a given microorganism of interest is likely to infect chimpanzees depends, however, on a second factor: how much is known about the microorganisms' biology and, more critically, how much is known about their genetic diversity and the distribution thereof amongst their hosts. Where host-parasite co-evolution is already known to have resulted in co-divergence (host divergence drives parasite divergence) and/or marked host-specificity (parasites infecting a given host species are more closely related with each other than to any other parasites), even low microorganism detection rates can be trusted to mostly reflect chimpanzee infection. A good example here is simian immunodeficiency viruses infecting chimpanzees (SIVcpz). Faecal sampling-based molecular analyses identified some wild communities with low detection rates (Keele *et al.*, 2006; Li *et al.*, 2012; Van Heuverswyn *et al.*, 2007). However, since SIVcpz has repeatedly and only been found in chimpanzees (Sharp and Hahn, 2010), including some natural infections documented from chimpanzee tissues (reviewed in Etienne *et al.* (2011)), there is no reason to suspect that these low detection rates reflect anything else than low faecal prevalence. Here, controlling for diet-induced microorganism detection is unnecessary. On the contrary, in cases where microorganisms are known to frequently undergo host switches and/or to simultaneously exploit several host species or when nothing is known about the association of these microorganisms and their host(s), detection of microorganisms from prey should be ruled out to reinforce the belief in an association between the newly detected microorganism and chimpanzees. A good example of this is a recent study that investigated the genetic diversity of polyomaviruses infecting NHP (Scuda *et al.*, 2013). This study made use of the same set of chimpanzee faecal samples from Loango that were examined here. Only 3% of the samples tested positive and among these a

single one contained a sequence, which could be related to a group consisting of two human viruses (BK and JC viruses) and several viruses infecting cercopithecines, i.e. potential prey of Loango chimpanzees. Significant host-parasite co-divergence of polyomaviruses and their mammal hosts was recently ruled out (Tao *et al.*, 2013). The (uncertain) phylogenetic placement of this sequence cannot be used as an argument in favor of its chimpanzee origin. In this context, the fact that the faecal sample from which this novel polyomavirus was identified was negative for prey is the main (positive) experimental evidence linking this virus to chimpanzees.

The high prey detection rate/prevalence that we found here may point towards a high rate of false positives, which may result from laboratory or environmental contamination. Multiple measures were taken *a priori* to avoid laboratory contamination: i) the laboratory in which experiments were performed consists of separated pre- and post-PCR facilities, ii) any PCR product generated in this laboratory over the last 3 years comprised dUTP and UNG was systematically added to reactions for this study (to remove PCR product contaminants), iii) multiple PCR systems were used. *A posteriori*, several lines of evidence point towards a low rate of laboratory work-induced false positives: i) Taï chimpanzees are known to prey on the mammal species detected (Boesch and Boesch-Achermann, 2000); ii) prey detection rates in Taï were in line with the observation of higher hunting frequency in October-November (Boesch and Boesch-Achermann, 2000); iii) out of 15 Taï samples found positive for red colobus, 8 different haplotypes were identified; and iv) haplotype distribution through time was not-random and was possibly compatible with discrete collective hunting events. Altogether, laboratory contamination is unlikely to have affected our prey prevalence estimates.

Environmental contamination of the samples cannot be ruled out by laboratory analysis. Two gorilla samples collected on the same day (out of a total of 37 possible collection days) were shown to contain forest elephant DNA, which points towards some degree of sample contamination in the field (elephant density in Loango is high (Head *et al.*, 2013; Morgan, 2007)). We note that the blocking system we used was extremely efficient at blocking gorilla DNA: after sequencing, only 3.2% of the gorilla samples turned out to be gorilla positive whereas 62.6% of the Loango chimpanzee samples and 69.2% of the Taï chimpanzee samples were found to be chimpanzee positive. Gorilla DNA is therefore unlikely

to have masked further environmental contaminants, meaning that environmental contamination may concern about 2% of the samples in total. This would only account for a minor fraction of prey prevalence in chimpanzees. We are therefore confident that our estimates of prey prevalence mostly reflect excretion after ingestion.

Checking that the diet of predatory apes does not influence the outcome of microorganism molecular epidemiology based on faecal sampling implies making technical decisions that will have their importance. Our recommendation would be to initially favor undirected approaches using blocking primers: these do not only moderate the risk of contamination but also offer greater flexibility as they do not require any prior information on the diet of predatory apes, which in many cases is poorly understood. A drawback is that they will likely underestimate prey prevalence, as shown here with Taï chimpanzees. Better assessment of prey prevalence could then be obtained by designing specific systems targeting prey identified using undirected approaches. Alternatively, the correction factor determined here for Taï chimpanzees may be used for extrapolation (as we did for Loango chimpanzees). It is important to bear in mind that controlling for prey DNA cannot entirely exclude a prey origin of the microorganism. Microorganism DNA could possibly outlive prey DNA in the digestive tract due to a slower decay rate or a temporary non-viable infection (Paula *et al.*, 2015).

It would be useful for further sites where predatory ape diet is well-characterized to use comparable methodologies as we did here, as it will help to determine whether results obtained in Taï can be further generalized. We also note that molecular analysis of prey in faeces could serve as a useful and sensitive complementary tool for the study of predatory ape hunting culture and diet, which currently mostly relies on direct observations (but see recent developments using stable isotopes; (Fahy *et al.*, 2013)). Our data also provide evidence that where prey populations are large and genetically diverse, CR haplotyping may be used to distinguish different hunting events and shared meals and may therefore allow tackling the question of hunting frequency.

4.5 Conclusion

In this chapter, we demonstrate that predatory ape faecal samples often test positive for prey DNA. Depending on microorganism faecal prevalence and the extent of prior knowledge about host-parasite associations, this should prompt microbiologists, parasitologists and virologists who use faecal samples to investigate the diversity of predatory ape parasites, to consider dietary items as a possible source of contamination; in a number of cases specific controls should be included in the experimental design. Finally, we would like to highlight that such controls would contribute to further integrating epidemiological and behavioural research on predatory apes (Gogarten *et al.*, 2014), as the data generated will both help in identifying meaningful predatory ape-microorganism associations and in describing their diets.

CHAPTER 5

GENERAL DISCUSSION AND PERSPECTIVES

The results presented in this study show that using faecal samples can be suitable and efficient, not only to address broad-scale questions such as those of the geographic distribution and genetic diversity of great ape malaria parasites, but also to address small-scale epidemiological questions which might, in comparison, require a higher (finer-scale) resolution and thus more sensitive screening. Faecal screening is expected to be less sensitive compared to blood screening for detection of malaria parasitaemia. The extent (quantity, frequency) to which *Plasmodium* DNA is shed in great ape faeces is unknown, but it is largely recognized that in general faecal DNA is of lower quality due to more important and rapid nucleic acid degradation and fragmentation (Frantzen *et al.*, 1998; Taberlet *et al.*, 1999). Nevertheless, the overall methodology used in the present work conferred sufficient sensitivity (i.e. the probability of detection given that an individual is infected) to permit detection of epidemiological patterns of infection.

First of all, a high sample quality was ensured in the field by sampling chimpanzees that are habituated to humans, thus allowing collection of samples immediately after defecation, and adequate preservation by freezing on the same day at a temperature below -80°C.

Secondly, the reasoning behind the choice of the molecular methods for faecal screening of malaria parasite DNA was to maximise the sensitivity of the tests. Given the nature of the questions to be answered and the relatively low sample sizes, it was considered essential to reduce the odds of missing positive samples in order to improve the likelihood of detecting existing infection patterns. To achieve this, the initial screening was performed by qPCR amplification of very short mitochondrial DNA fragments (90 bp) as opposed to the much longer fragments targeted in faecal samples from other studies (e.g. 956 bp in Liu *et al.* (2010) and 704 bp in Prugnolle *et al.* (2010)). Subsequently, a maximum of effort was made to confirm the positives obtained. This was done by sequencing of larger fragments (preceded by cloning when necessary), and in addition, for the second dataset (pregnancy dataset), by excluding false positives like *Hepatocystis* sp. DNA from dietary origin by confirmatory standard PCR assays and control for contamination with monkey DNA. It is only during the analysis of the second dataset that *Hepatocystis* sp. sequences were obtained for the first time, making us aware about the possibility of false positives from dietary origin, and leading to the subsequent choice of methodology applied to this dataset. Contamination from dietary origin was thus not tested for in the first dataset (age dataset, Chapter 2), but an a posteriori control made by replicating the statistical analysis

after excluding all potential false positives from this dataset (i.e. all positive qPCR samples for which no sequences were obtained) confirmed the main findings on the effect of age.

Unfortunately it is not possible to directly compare sensitivity of this study's non-invasive methods with those from previously published work (i.e. Liu *et al.* (2010), Prugnolle *et al.* (2010)), as these were performed on different wild ape communities with probably different real infection rates, and these rates are unknown. Liu *et al.* (2010, 2014) estimated sensitivity by using the proportion of positive samples from positive individuals tested several times in one same day. This could not be done in the present work due to the lack of such samples.

Differences in the overall methodologies, including sample quality and diagnostic tests, and unknown sensitivities, make it difficult to compare faecal detection rates and estimated prevalence of infection of the TCP chimpanzee community with those obtained from other chimpanzee populations in other studies. The rate of faecal malaria parasite detection represented by the proportion of strictly confirmed (sequenced) positive samples, found in the TCP chimpanzee community was 24 %. This is based on the age dataset, which is representative of all sexes and most age classes of the studied group. This falls in the range of previously published faecal detection rates, which vary from 0 % to 67 % for various chimpanzee field sites (Boundenga *et al.*, 2015; Krief *et al.*, 2010; Liu *et al.*, 2010; Prugnolle *et al.*, 2010), and from 16 % to 24 % for the different chimpanzee subspecies (Liu *et al.*, 2010).

The proportion of positive individuals, when each individual is randomly sampled once (by bootstrap analysis with 1000 replicates), provides a one point prevalence estimate for the TCP study group, i.e. 33% (95% CI: 15% - 50%) and the proportion of individuals who tested positive at least once during the ca. 2 months sampling period (by bootstrap analysis with 1000 replicates) provides an estimate of the period prevalence, i.e. 80% (95% CI: 60% - 95 %). The point prevalence rate found in this study, and thus in western chimpanzees, falls in the range of those estimated non-invasively by Liu *et al.* (2010) in the three other chimpanzee subspecies and in western lowland gorillas (32% to 48%), although, again, these estimates are difficult to compare directly due to differences in methodology. Those provided by Liu *et al.* (2010) were corrected for repeat sampling (making them thus comparable to point prevalence), but were, unlike ours, also corrected for sensitivity of the

diagnostic test. Assuming a lower sensitivity of faecal detection compared to blood detection, the estimates provided in the present study most certainly represent underestimates of the real prevalence levels of infection. Nevertheless, our results confirm that, like in other chimpanzee subspecies and western gorillas, *Plasmodium* prevalence rates and diversity in western chimpanzees are high.

We also confirmed the presence of the three chimpanzee *Laverania* species as well as *P. vivax*-like parasites (all previously described in *P. t. verus* except for *P. billcollinsi*). As previously found by Liu *et al.* (2010), *Laverania* species predominate, representing 95 % of all sequences obtained in this work (65 %, 11% and 6 % for *P. gaboni*, *P. billcollinsi* and *P.* reichenowi, respectively).

Mixed species infections were detected in only 5.6 % of all the sequenced samples. However, given previously published findings(Boundenga *et al.*, 2015; Krief *et al.*, 2010; Liu *et al.*, 2010), it is likely that mixed infections occur at a higher frequency than detected here. As will be discussed below (see page 86), the presence of one parasite species in a host could potentially decrease the parasite density of another species (or even block its presence), through resource competition or host immune system (Graham, 2008; Rynkiewicz *et al.*, 2015). Some *Plasmodium* parasites might dominate others during co-infections and thereby occult their presence or render their detection challenging. This seems for instance to be the case for *P. falciparum* towards *P. vivax* in humans (Mayxay *et al.*, 2004) and had already been observed by Rodhain (1936) in experimental chimpanzees. By targeting longer DNA fragments and using single genome amplification methods, Liu *et al.* (2010) identified and characterized co-infections with different *Plasmodium* species in the majority of their positive samples.

The impression given by the high prevalence levels found, especially assuming that they are underestimates of real infection rates, is that most individuals are probably constantly or at least very frequently infected by malaria parasites. Actually, just like in humans from highly endemic areas where most people seem to repeatedly carry low grade infections, it is likely to be more often increased parasitaemia that are detected rather than the presence or absence of infections (Leke *et al.*, 2010).

It cannot be excluded that, as previously documented in mice (Abkallo *et al.*, 2014) and macaques (Kawai *et al.*, 2014), a fraction of the positive faecal results reflect pre-

erythrocytic stages rather than blood stages. However, at least for the *Plasmodium* species, it is highly probable that most of the time positive faecal samples reflect blood stages. All the *Plasmodium* species found in AGA so far seem to be host specific with no other reservoir species to ensure transmission. Moreover, as discussed in chapter 2, the low chimpanzee density of the research area suggests that transmission mainly occurs within groups, and group sizes are small. It seems thus reasonable to expect that, in order to ensure transmission, erythrocytic stages are frequent in these chimpanzees. This is, though, unless all the *Plasmodium* species infecting these chimpanzees are characterized by long-lasting quiescent liver stages, which would then not require the same intensity of re-infections to maintain infection in that population. The unique sample which was confirmed positive for *Hepatocystis sp.* but negative for monkey DNA, on the other hand, is more likely to reflect a liver stage or direct clearance after incidental inoculation, as infection with this parasite, known to infect monkeys and bats (Ayouba *et al.*, 2012; Schaer *et al.*, 2013), was never found in chimpanzees so far. The possibility that the faecal *Hepatocystis* DNA was from dietary origin and outlived the prey (monkey) DNA also remains.

Instead of increased parasite density or frequency of infection, physiological increase of certain body fluids in the faeces could also be hypothesized in order to explain increased detection of *Plasmodium* DNA in younger individuals and pregnant females. Yet, this explanation could be excluded by controlling for the quantity of mammal DNA in the faeces.

Despite the fact that knowledge is still very limited and that findings must be interpreted with caution, it seems thus reasonable to state that the patterns of faecal malaria parasite detection apparent in this work reflect changes in the frequency of new infections and/or in parasite densities, induced by variation of the susceptibility to these parasites. As discussed in chapters 2 and 3, immunity and/or the presence of a placenta associated with the host condition (age and pregnancy status) most probably play a role in these changes of susceptibility. However, variations in the exposure to the vectors and thus to malaria parasites might also occur and play a role. It was for instance demonstrated that pregnant women are more attractive to *Anopheles* mosquitoes compared to non-pregnant women, probably for physiological reasons (i.e. increased exhaled breath and abdominal temperature) (Ansell *et al.*, 2002; Lindsay *et al.*, 2000). Also to consider are possible behavioural changes that could lead to increased exposure such as different nesting habits.

Moreover, an important aspect to highlight when discussing the mechanisms underlying changes in parasite infection and/or densities is that in a given host, malaria parasites are part of a parasite community (note that in this paragraph the term parasite is used in its broad ecological sense, designating micro and macroparasites) (Faure, 2014). This implies that, just like in an ecosystem, multiple and complex interactions in between these parasites as well as with their host (i.e. their environment) take place, which can also partly dictate the presence of parasite species or their densities in that host, as opposed to a single host-parasite relationship (Pedersen and Fenton, 2007; Rynkiewicz *et al.*, 2015), and perhaps play a role in the patterns of malaria parasite detection described in chapter 2 and 3. Telfer *et al.* (2010) actually demonstrated for the first time in a natural population that co-infection explains more variation in the risk of infection compared to other factors such as host condition (e.g. age) and exposure risk (e.g. season) (Telfer *et al.*, 2010). Co-infection interactions can take place via competition for shared resources (e.g. nutrients, physical site of infection) or the immune system of the host (e.g. cross-reactivity, immunosuppression) (Graham, 2008; Pedersen and Fenton, 2007; Rynkiewicz *et al.*, 2015). A meta-analysis of data from co-infections in humans actually suggests that the mechanisms of parasite interaction are mainly resource-mediated (Griffiths *et al.*, 2014). This would be likely to occur between haemoparasite species, which target the same cells, like erythrocytes. For instance, *Babesia microti* infection in wild voles (*Microtus agrestis*) negatively affects host susceptibility to *Bartonella* spp. infection, and vice versa (Telfer *et al.*, 2010). Likewise, a negative association between *Plasmodium* spp. and *Babesia* sp. has been recently observed in wild Malagasy primates (*Propithecus verreauxi*), where high *Babesia* sp. infection levels in younger individuals seems to confer a protection against *Plasmodium* spp. infections, possibly via shared resource competition (A. Springer, personal communication). Inhibition of *P. cynomolgi* infection by *B. microti* was also previously described in experimental rhesus macaques (van Duivenvoorde *et al.*, 2010; Voorberg-vd Wel *et al.*, 2008). Furthermore, malaria parasites and various helminth species have been shown to interact in humans, with either antagonistic or beneficial effects on each other, possibly mediated by cross-reactive immune responses (Faure, 2014). In order to fully understand the patterns of malaria infection in AGA, it would thus be necessary to also explore and take into account co-infections with other parasite strains and species.

The patterns found in the TNP study population of wild chimpanzees are strikingly similar to those found in human populations from malaria endemic areas: susceptibility to malaria parasites is higher in younger individuals and during pregnancy. These are the first signs that epidemiological characteristics of AGA malaria are comparable to those in humans and encourage further research to explore the influence of other known intrinsic factors such as co-infections with potentially immunosuppressant pathogens (e.g. SIV), and genetic factors. Increased susceptibility in younger individuals has, since then, also been observed in western lowland gorillas (Mapua *et al.* 2015).

The resemblance with human patterns also naturally raises the question whether younger individuals and pregnant females are, like in humans, more prone to develop clinical malaria. So far, pathogenicity has never been demonstrated in chimpanzees in natural conditions, and healthy non-pregnant adults probably benefit from a certain degree of acquired clinical immunity, but it would be reasonable to think that malaria infection would start having some level of detrimental effects when the host's ability to control parasite density is diminished. Higher levels of blood parasitaemia are likely to at least induce higher degrees of anaemia (Kotepui *et al.*, 2015; Oni and Oguntibeju, 2006), which in turn could cause morbidity or even mortality. Anaemia is actually recognized as one of the major cause of morbidity and mortality related to malaria in young children and pregnant women (Ekvall *et al.*). Also, if placental malaria does actually occur in chimpanzees, it would be difficult to conceive that this would be without the slightest consequence on pregnancy outcome or child health. Deleterious effects on pregnancy outcome and/or infant health would also mean a general impact on population reproductive success and fitness, thereby shaping population dynamics.

On the other hand, phylogenetic analyses suggest that AGA might historically have been hosting *Laverania* malaria parasites before humans, who were infected by *P. falciparum* through a host switch event from gorillas (Liu *et al.*, 2010). This would imply a host-parasite relationship at a more advanced evolutionary state, with possibly better adaptation and ability of the host to control the infection and/or its effects, and perhaps modified virulence of the parasite. Yet, an evolutionary trend toward decreasing virulence as a result of the age of the host–parasite relationship has not been demonstrated for malaria parasites so far (Escalante *et al.*, 1998).

In any case, further studying the relationship between malaria parasites and chimpanzees in natural conditions is important as it will provide further information on the significance of malaria infection for wild great ape populations' health, and can serve as model to understand the host-parasite relationship in human populations, where malaria is believed to have acted as an important driver of recent evolution (Carter and Mendis, 2002; Sabbatani *et al.*, 2010).

Pathogenicity of malaria parasites in great apes can be investigated indirectly by, for instance, studying genetic factors which could eventually point towards a certain level of pathogenicity and thus evolutionary disadvantage exerted by malaria infection leading to selective pressure, or directly by searching for clinical signs. The present work's findings show that younger individuals and pregnant females would constitute ideal target groups to search for signs of clinical malaria in great apes. The likelihood of finding positive individuals would be optimized, an important benefit considering that collecting samples and health data from wild chimpanzees is extremely laborious. Moreover, as already discussed, these groups might be more vulnerable to clinical malaria, which would also improve chances of detecting any pathological effect. Investigating the occurrence of clinical malaria could be done non-invasively by applying similar malaria detection methods to those used in this work, as well as non-invasive ways to measure various clinical parameters, such as estimating body temperature via faecal temperature (method already established in chimpanzees by Jensen *et al.* (2009)), measuring haemoglobin and inflammation markers in urine samples and recording illness behaviour.

It would of course be extremely useful to also investigate the sensitivity of the detection methods and the duration of infections to have a better understanding of what the presence of malaria parasite DNA in faeces actually reflects. This could, for instance, be done by analysing paired blood and faecal samples from sanctuary apes and/or perform longitudinal studies in wild apes. It would eventually permit more accurate estimations of real prevalence of infection, and help with interpreting results.

Assessing the impact of infection on social behaviour such as hierarchical status would also be a way to search for signs of virulence, indirectly.

Important to note is that the non-invasive methods can also be applied to study the effects of extrinsic factors on malaria infection such as group size, nesting habits, climate etc. and that they render comparison with other ape populations feasible, which would constitute an essential undertaking to improve general understanding of all the above-mentioned aspects.

Finally, the non-invasive work performed on great ape malaria in this study did not only constitute a means to acquire knowledge on epidemiological aspects of malaria in great apes, but also served as a model to help identify general methodological difficulties or challenges associated with faecal epidemiology of infectious agents, which is particularly important since faecal epidemiology is used increasingly broadly. In the present case, it puts forward the danger of contamination with microorganisms of dietary origin, which could act as a confounding factor when testing for pathogen infections, and leads to suggestions on how to control for this. New studies which rely on faecal samples to describe the diversity of microorganisms infecting wild primates are constantly published (e.g. the viral diversity in wild great apes, (Seimon *et al.*, 2015)). However, dietary contamination is usually not taken into account. Emphasizing this risk is thus important and will hopefully contribute to the improvement of methods and benefit the overall quality of research in this field.

SUMMARY

Amongst infectious diseases, malaria represents a major public health concern. It is geographically widespread and the yearly toll of clinical cases and mortalities triggered by malaria parasite (*Plasmodium* spp.) infections in human populations living in tropical and subtropical areas is heavy. Several malaria parasites also infect African great apes (AGA). These are subject to continuous interest, given that their hosts are our closest phylogenetic relatives, and that they are morphologically and genetically very close to the human borne malaria parasites. Research has been driven mainly by the idea that malaria in AGA can serve as model for human malaria, as well as by the concern of zoonotic transmission. These parasites were initially studied in captive or experimental animals, but over the past decades they have also been increasingly investigated in wild ape populations. This was mainly rendered possible by the use of molecular diagnostic tools, as well as, more recently, by the discovery that malaria parasites can be detected non-invasively (i.e. in faeces). So far, most of the research effort has been put into the analysis of the parasite diversity, distribution, and phylogenetic relationships with human parasites. Even if information is still scarce, some research has also been carried on the identification of vectors and the possibilities of cross-transmissions to humans. Epidemiological and biological questions such as the determinants of malaria parasite infection and the pathogenicity of malaria parasites in AGA, however, have been left largely unexplored and the question of what malaria infection actually implies in terms of chimpanzee populations' health remains open.

This work was thus undertaken in the context of a broader study aiming at creating baseline epidemiological and biological data on malaria parasite infection in wild AGA, starting with the intrinsic determinants of susceptibility to malaria parasite infection in wild chimpanzees, our closes relatives. More specifically, the objective was to investigate the effects of young age and pregnancy, both well-known risk factors for malaria in humans in malaria endemic areas, on malaria parasite infection in wild chimpanzees, and to do so by using faecal samples to detect infection. An additional aim of this work was also to explore the practicality of studying such fine scale epidemiological questions in wild apes with non-

invasive methods, as this was never reported before, and to share the resulting experience with the hope to contribute to the improvement of such methods.

The first chapter gives a review of the current knowledge on malaria parasite infection in wild AGA, and the use of non-invasive samples to study microorganisms infecting great apes, with particular focus on malaria parasites.

The second and third chapters describe the studies on the effects of age and pregnancy on malaria parasite infection in wild chimpanzees. Both studies were performed in the Taï National Park, Côte d'Ivoire, on a wild western chimpanzee (*Pan troglodytes verus*) population of which several groups have been habituated by the Taï Chimpanzee Project (Max Planck Institute for Evolutionary Anthropology, Leipzig) since more than 35 years. One hundred and forty-one faecal samples from 19 individuals ranging from 3 to 47 years old, and 384 samples collected during 40 pregnancies and 36 corresponding post-pregnancies were tested for malaria parasites in order to explore the influence of age and pregnancy respectively. Faecal samples used in this study were collected straight after defaecation from identified individuals, and preserved frozen until analysis. They were gathered during one year of fieldwork in the frame of this doctoral study, as well as during the 12 preceding years through a collaborative health project between the Taï Chimpanzee Project and the Robert Koch Institute, Berlin, and represent thus a unique set of data. Samples were analysed by polymerase chain reaction, sequencing, and cloning when necessary. Data were analysed using generalized linear mixed models. Based on the faecal detection rate, the point prevalence of malaria parasite infection in the study population was estimated at 33 %. In both studies, faecal detection rates decreased with age, suggesting that individuals acquire protective immunity through continuous exposure. Moreover, probability of detection increased towards the end of pregnancy, which could, for instance, be linked to pregnancy-related hormonal and immune alterations, and/or infection with parasites which specifically target the placenta. These results highlight similar dynamics of malaria parasite infection in humans and chimpanzees. Susceptibility to malaria infection seems to be enhanced in young individuals and pregnant females, which further raises questions on the effects of such infections on child mortality/morbidity and pregnancy outcome. It appears that young individuals and pregnant females would constitute ideal target groups for the exploration of malaria parasite pathogenicity in wild chimpanzees. The outcome of these analyses also

demonstrates that faeces can constitute a suitable substrate to investigate small-scale epidemiological patterns of malaria parasite infection. It is, however, essential to improve the understanding of what faecal detection of malaria parasite DNA truly reflects in order to facilitate results interpretation.

In this line, molecular analysis performed during the course of this work allowed us to pinpoint an important issue (i.e. dietary contamination) to address when using faecal samples to screen for infectious agents. This led to a technical and advisory study on the use of faecal samples to study pathogens and other parasites (viruses, prokaryotes or eukaryotes) infecting chimpanzees, described in chapter 4. Faecal samples are an important source of information on parasites infecting wild great apes. Molecular analysis of faecal samples has already been used for deciphering the origins of major human pathogens such as HIV-1 or *Plasmodium falciparum*. However, for apes that hunt (chimpanzees and bonobos), detection of parasite nucleic acids may reflect either true infection of the host of interest or ingestion of an infected prey, e.g. another non-human primate. Consequently, there is a risk of confusing parasites infecting prey with those infecting these predatory great apes. To determine the potential magnitude of this issue, we estimated the prevalence of prey DNA in faecal samples obtained from two wild chimpanzee communities. We observed values >15 %, which are higher than or close to the faecal detection rates of many great ape parasites. Contamination of faecal samples with parasite DNA from dietary origin may therefore occasionally impact non-invasive epidemiological studies. This problem can be addressed (at least partially) by monitoring the presence of prey DNA. We therefore propose that diet analyses be incorporated in the set of controls already applied in the field and discuss cases where such controls will reveal necessary.

In chapter 5, the conclusions of the present work are discussed, with special focus on the lessons learned about the non-invasive methodology, the interpretation of the epidemiological outcomes, as well as the perspectives for methodological improvement and application of non-invasive approaches to further investigate the epidemiology and biology of malaria parasites in AGA.

SAMENVATTING

Malaria is één van de belangrijkste infectieziekten voor de volksgezondheid. De ziekte heeft een zeer ruime geografische verspreiding en de jaarlijkse tol van klinische gevallen en sterfte veroorzaakt door malariaparasieten (*Plasmodium* spp.) is zeer groot bij de menselijke populaties in tropische en subtropische gebieden. Verschillende malaria species infecteren ook Afrikaanse mensapen (AGA). Deze wekken veel interesse op omdat hun gastheren onze meest nabije fylogenetische verwanten zijn, maar ook omdat ze morfologisch en genetisch zeer dicht bij de malaria species van de mens staan. Onderzoek naar malaria bij AGA is vooral gedreven door het idee dat deze kan dienen als model voor menselijke malaria, maar ook door de bezorgdheid van mogelijke zoönotische transmissie. Aanvankelijk werden deze parasieten onderzocht bij dieren in gevangenschap of in experimentele condities, maar in de afgelopen decennia werden zij ook in toenemende mate onderzocht bij populaties van wildlevende apen. Dit werd mogelijk gemaakt door het gebruik van moleculaire diagnostische hulpmiddelen, alsmede, recent, door de ontdekking dat malaria met niet-invasieve technieken kan worden gedetecteerd, namelijk in feces. De meeste aandacht van onderzoek over malaria bij AGA ging tot nu toe naar de parasiet diversiteit, de verspreiding en de fylogenetische verwantschappen met humane parasieten. Er werd ook onderzoek gedaan naar de identificatie van de vectoren en de mogelijkheid van cross-transmissie naar mensen, alhoewel de informatie hierover nog beperkt is. Epidemiologische en biologische vragen, zoals de determinanten van de malariaparasiet infectie en de pathogeniciteit van malariaparasieten bij AGA, zijn grotendeels onontgonnen gebied; en de vraag wat malaria-infectie daadwerkelijk inhoudt voor de gezondheid van chimpansee populaties blijft open.

Dit werk werd ondernomen in het kader van een breder onderzoek dat als doel heeft om basis epidemiologische en biologische gegevens over malaria infecties bij wilde AGA te genereren, te beginnen met de intrinsieke determinanten van de vatbaarheid voor malaria infecties bij wilde chimpansees, onze dichtste familie. Meer in het bijzonder was onze doelstelling om de effecten van jonge leeftijd en dracht op malaria infecties bij wildlevende chimpansees te onderzoeken, door middel van onderzoek op fecale monsters om infectie te detecteren. Jonge leeftijd en zwangerschap zijn namelijk bekende risicofactoren voor malaria bij de mens in malaria endemische gebieden. Een bijkomende doelstelling van dit

werk was om de uitvoerbaarheid te onderzoeken van de studie van dergelijke gedetailleerde epidemiologische vragen bij wilde apen, door gebruik te maken van niet-invasieve methoden, aangezien dit niet eerder werd gerapporteerd, en de verkregen ervaring te kunnen delen met de hoop te kunnen bijdragen aan het verbeteren van zulke werkwijzen.

Het eerste hoofdstuk geeft een overzicht van de huidige kennis over malaria infecties bij wilde AGA, en het gebruik van niet-invasieve monsters om micro-organismen die mensapen infecteren te bestuderen, met bijzondere aandacht voor malaria parasieten.

De tweede en derde hoofdstukken beschrijven de studies over de effecten van leeftijd en zwangerschap op malaria infecties bij wilde chimpansees. Beide studies werden uitgevoerd in het Taï Nationaal Park, Ivoorkust, op een populatie West-Afrikaanse chimpansees (*Pan troglodytes verus*), waarvan verschillende groepen zijn gewend gemaakt door het Taï Chimpansee Project (Max Planck Instituut voor Evolutionaire Antropologie, Leipzig), sinds meer dan 35 jaar. Respectievelijk, 141 fecesmonsters van 19 individuen tussen 3 en 47 jaar oud, en 384 monsters genomen gedurende 40 zwangerschappen en 36 corresponderende periodes na de zwangerschap werden verzameld en getest op malariaparasieten om de invloed van leeftijd en zwangerschap te onderzoeken. Fecesmonsters werden onmiddellijk na defecatie van geïdentificeerde individuen verzameld, en ingevroren tot ze geanalyseerd werden. Deze monsters werden in de loop van een jaar veldwerk verzameld in het kader van dit doctoraatsonderzoek, evenals tijdens de 12 voorgaande jaren van een samenwerkingsproject tussen het Taï Chimpansee Project en het Robert Koch Instituut, Berlijn over de gezondheid van chimpansees. Deze monsters vormen zo een unieke dataset. De monsters werden geanalyseerd door middel van de polymerase kettingreactie (PCR), en indien nodig door sequentiebepaling en klonering. De gegevens werden geanalyseerd met behulp van veralgemeende lineaire gemengde modellen (GLMM). Gebaseerd op de detectiegraad in fecesmonsters werd de puntprevalentie van malaria infecties in de studiepopulatie geschat op 33%. In beide studies verminderde de fecale detectiegraad met de leeftijd wat suggereert dat individuen een beschermende immuniteit ontwikkelen als gevolg van continue blootstelling aan infectie. Bovendien steeg de probabiliteit van infectie naar het einde van de dracht, wat mogelijk gekoppeld kan zijn aan dracht-gerelateerde hormonale en immunologische veranderingen en/of infectie met parasieten die zich

specifiek op de placenta richten. Deze resultaten tonen een vergelijkbare dynamiek van malaria infecties aan bij mensen en chimpansees. De gevoeligheid voor een malaria-infectie lijkt verhoogd te zijn bij jonge individuen en drachtige vrouwelijke dieren, wat vragen oproept met betrekking tot het effect van dergelijke infecties op sterfte/morbiditeit bij jonge individuen en op de uitkomst van de dracht. Het lijkt erop dat jonge individuen en drachtige vrouwelijke dieren de ideale doelgroepen zijn om de pathogeniciteit van malariaparasieten bij wilde chimpansees te onderzoeken. Het resultaat van deze analyses toont ook aan dat feces een geschikt substraat kan vormen om kleinschalige epidemiologische patronen van malaria infecties te onderzoeken. Het is echter noodzakelijk om de waarde van fecale detectie van malariaparasiet DNA te evalueren om de interpretatie van de resultaten te vergemakkelijken.

In deze lijn, konden we aan de hand van de moleculaire analyses uitgevoerd op de fecale monsters een belangrijk probleem identificeren, nl de verontreiniging door het dieet van de chimpansees. Dit leidde tot een technische en adviserende studie over het gebruik van fecesmonsters voor de identificatie van pathogenen en parasieten (virussen, prokaryoten of eukaryoten) die chimpansees infecteren; dit wordt beschreven in hoofdstuk 4. Fecesmonsters zijn een belangrijke bron van informatie over parasitaire infecties van wilde mensapen. Moleculaire analyse van fecesmonsters is reeds gebruikt voor het onderzoek naar de oorsprong van belangrijke humane pathogenen zoals HIV-1 en *Plasmodium falciparum*. Echter, voor apen die jagen (o.a. chimpansees en bonobo's), kan de aanwezigheid van parasiet nucleïnezuren in feces wijzen op een infectie van de gastheer zelf, maar ook op de predatie van een geïnfecteerde prooi, zoals bijvoorbeeld een andere niet-menselijke primaat. Er bestaat derhalve een risico voor verwarring tussen een werkelijke parasitaire infectie bij de predaterende mensaap en een infectie van zijn prooi. Om de potentiële omvang van dit probleem te bepalen, hebben we de prevalentie van prooi DNA in fecale monsters bij twee gemeenschappen van wilde chimpansees geschat. Waarden van meer dan 15% werden waargenomen, wat hoger of in de buurt ligt van de fecale detectiegraad van parasieten bij mensapen. Er moet dus rekening gehouden worden met het feit dat contaminatie van fecale monsters met parasiet DNA, afkomstig van de prooi in sommige gevallen een invloed kan hebben op epidemiologische studies die gebruik maken van niet-invasieve methodes. Dit probleem kan (ten minste gedeeltelijk) worden aangepakt

door het monitoren van de aanwezigheid van prooi DNA in het feces monster. We stellen daarom voor om dieetanalyse op te nemen in de controles die reeds worden toegepast in het veld en dat gevallen waarbij zulke controles nodig blijken te zijn worden besproken.

In hoofdstuk 5, worden de conclusies van het huidige werk besproken, met speciale aandacht voor de lessen geleerd over het gebruik van de niet-invasieve methodes, de interpretatie van de epidemiologische resultaten, evenals de perspectieven voor methodologische verbeteringen en toepassing van niet-invasieve benaderingen om de epidemiologie en biologie van malariaparasieten bij AGA verder te onderzoeken.

REFERENCES

Abkallo, H.M., Liu, W., Hokama, S., Ferreira, P.E., Nakazawa, S., Maeno, Y., Quang, N.T., Kobayashi, N., Kaneko, O., Huffman, M.A., Kawai, S., Marchand, R.P., Carter, R., Hahn, B.H., Culleton, R., 2014. DNA from pre-erythrocytic stage malaria parasites is detectable by PCR in the faeces and blood of hosts. Int. J. Parasitol. doi:10.1016/j.ijpara.2014.03.002

Adler, S., 1923. Malaria in chimpanzees in Sierra Leone. Ann. Trop. Med. Parasitol.. 17, 13–19.

A-Elgayoum, S.M.E., El-Rayah, E.-A., Giha, H.A., 2010. Towards a noninvasive approach to malaria diagnosis: detection of parasite DNA in body secretions and surface mucosa. J. Mol. Microbiol. Biotechnol. 18, 148–55. doi:10.1159/000308516

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J Mol Biol 215, 403–410. doi:10.1016/S0022-2836(05)80360-2

Anderson, D.P., Nordheim, E. V., Moermond, T.C., Gone Bi, Z.B., Boesch, C., 2005. Factors Influencing Tree Phenology in Tai National Park, Cote d'Ivoire1. Biotropica 37, 631–640. doi:10.1111/j.1744-7429.2005.00080.x

Ansell, J., Hamilton, K.A., Pinder, M., Walraven, G.E., Lindsay, S.W., 2002. Short-range attractiveness of pregnant women to Anopheles gambiae mosquitoes. Trans R Soc Trop Med Hyg 96, 113–116.

Ayouba, A., Mouacha, F., Learn, G.H., Mpoudi-Ngole, E., Rayner, J.C., Sharp, P.M., Hahn, B.H., Delaporte, E., Peeters, M., 2012. Ubiquitous Hepatocystis infections, but no evidence of Plasmodium falciparum-like malaria parasites in wild greater spot-nosed monkeys (Cercopithecus nictitans). Int J Parasitol 42, 709–713. doi:10.1016/j.ijpara.2012.05.004

Baayen, R.H., 2008. Analyzing linguistic data : a practical introduction to statistics using R. Cambridge University Press, Cambridge, UK ; New York.

Bardají, A., Sigauque, B., Sanz, S., Maixenchs, M., Ordi, J., Aponte, J.J., Mabunda, S., Alonso, P.L., Menéndez, C., 2011. Impact of malaria at the end of pregnancy on infant mortality and morbidity. J. Infect. Dis. 203, 691–9. doi:10.1093/infdis/jiq049

Barnett, O.E., Worobey, M., Holmes, E.C., Cooper, A., 2004. Detection of TT virus among chimpanzees in the wild using a noninvasive technique. J. Wildl. Dis. 40, 230–7. doi:10.7589/0090-3558-40.2.230

Barr, D.J., Levy, R., Scheepers, C., Tily, H.J., 2013. Random effects structure for confirmatory hypothesis testing: Keep it maximal. J Mem Lang 68. doi:10.1016/j.jml.2012.11.001

Bates, D., Maechler, M., Bolker, B., 2013. lme4: Linear mixed-effects models using S4 classes. R Packag. version 0.999999-2. 999999. doi:citeulike-article-id:1080437

Beadell, J.S., Fleischer, R.C., 2005. A restriction enzyme-based assay to distinguish between avian hemosporidians. J Parasitol 91, 683–685. doi:10.1645/GE-3412RN

Bejon, P., Mwangi, T.W., Lowe, B., Peshu, N., Hill, A.V.S., Marsh, K., 2008. Helminth infection and eosinophilia and the risk of Plasmodium falciparum malaria in 1- to 6-year-old children in a malaria endemic area. PLoS Negl. Trop. Dis. 2, e164. doi:10.1371/journal.pntd.0000164

Blacklock, B., Adler, S., 1922. A parasite resembling Plasmodium falciparum in a chimpanzee. Ann. Trop. Med. Parasitol. 160, 99–106.

Blanquart, S., Gascuel, O., 2011. Mitochondrial genes support a common origin of rodent malaria parasites and Plasmodium falciparum's relatives infecting great apes. BMC Evol Biol 11, 70. doi:10.1186/1471-2148-11-70

Blasse, A., Calvignac-Spencer, S., Merkel, K., Goffe, A.S., Boesch, C., Mundry, R., Leendertz, F.H., 2013. Mother-offspring transmission and age-dependent accumulation of simian foamy virus in wild chimpanzees. J Virol 87, 5193–5204. doi:10.1128/JVI.02743-12

Blinkova, O., Victoria, J., Li, Y., Keele, B.F., Sanz, C., Ndjango, J.-B.N., Peeters, M., Travis, D., Lonsdorf, E. V, Wilson, M.L., Pusey, A.E., Hahn, B.H., Delwart, E.L., 2010. Novel circular DNA viruses in stool samples of wild-living chimpanzees. J. Gen. Virol. 91, 74–86. doi:10.1099/vir.0.015446-0

Boesch, C., Boesch-Achermann, H., 2000. The Chimpanzees of the Taı¨ Forest: Behavioral Ecology and Evolution. Oxford University Press, Oxford.

Boessenkool, S., Epp, L.S., Haile, J., Bellemain, E.V.A., Edwards, M., Coissac, E., Willerslev, E., Brochmann, C., 2012. Blocking human contaminant DNA during PCR allows amplification of rare mammal species from sedimentary ancient DNA. Mol Ecol 21, 1806–1815. doi:10.1111/j.1365-294X.2011.05306.x

Boundenga, L., Ollomo, B., Rougeron, V., Mouele, L.Y., Mve-Ondo, B., Delicat-Loembet, L.M., Moukodoum, N.D., Okouga, A.P., Arnathau, C., Elguero, E., Durand, P., Liégeois, F., Boué, V., Motsch, P., Le Flohic, G., Ndoungouet, A., Paupy, C., Ba, C.T., Renaud, F., Prugnolle, F., 2015. Diversity of malaria parasites in great apes in Gabon. Malar. J. 14, 111. doi:10.1186/s12936-015-0622-6

Bouyou-Akotet, M.K., Adegnika, A.A., Agnandji, S.T., Ngou-Milama, E., Kombila, M., Kremsner, P.G., Mavoungou, E., 2005. Cortisol and susceptibility to malaria during pregnancy. Microbes Infect 7, 1217–1223. doi:10.1016/j.micinf.2005.04.008

Bouyou-Akotet, M.K., Ionete-Collard, D.E., Mabika-Manfoumbi, M., Kendjo, E., Matsiegui, P.-B., Mavoungou, E., Kombila, M., 2003. Prevalence of Plasmodium falciparum infection in pregnant women in Gabon. Malar. J. 2, 18. doi:10.1186/1475-2875-2-18

Brabin, B.J., 1983. An analysis of malaria in pregnancy in Africa. Bull World Heal. Organ 61, 1005–1016.

Brabin, B.J., Romagosa, C., Abdelgalil, S., Menéndez, C., Verhoeff, F.H., McGready, R., Fletcher, K.A., Owens, S., D'Alessandro, U., Nosten, F., Fischer, P.R., Ordi, J., 2004. The sick placenta-the role of malaria. Placenta 25, 359–78. doi:10.1016/j.placenta.2003.10.019

Bray, R.S., 1963. The Malaria Parasites of Anthropoid Apes. J. Parasitol. 49, 888–891.

Bray, R.S., 1960. Studies on malaria in chimpanzees. VIII. The experimental transmission and pre-erythrocytic phase of Plasmodium malariae, with a note on the host-range of the parasite. Am. J. Trop. Med. Hyg. 9, 455–465.

Bray, R.S., 1958a. Studies on malaria in chimpanzees. V. The sporogonous cycle and mosquito transmission of Plasmodium vivax shcwetzi. J. Parasitol. 44, 46–51.

Bray, R.S., 1958b. Studies on malaria in chimpanzees.VI. *Laverania falciparum.* Am. J. Trop. Med. Hyg. 7, 20-24.

Bray, R.S., Anderson, M.J., 1979. Falciparum malaria and pregnancy. Trans. R. Soc. Trop. Med. Hyg. 73, 427–431.

Broderick, C., Nadjm, B., Smith, V., Blaze, M., Checkley, A., Chiodini, P.L., Whitty, C.J.M., 2015. Clinical, geographical, and temporal risk factors associated with presentation and outcome of vivax malaria imported into the United Kingdom over 27 years: observational study. BMJ 350, h1703.

Brumpt, E., 1939. Les parasites du paludisme des chimpanzés. Comptes Rendus Soc. Biol. 130, 834–840.

Buppan, P., Putaporntip, C., Pattanawong, U., Seethamchai, S., Jongwutiwes, S., 2010. Comparative detection of Plasmodium vivax and Plasmodium falciparum DNA in saliva and urine samples from symptomatic malaria patients in a low endemic area. Malar. J. 9, 72. doi:10.1186/1475-2875-9-72

Calvignac-Spencer, S., Leendertz, S.A.J., Gillespie, T.R., Leendertz, F.H., 2012. Wild great apes as sentinels and sources of infectious disease. Clin. Microbiol. Infect. 18, 521–7. doi:10.1111/j.1469-0691.2012.03816.x

Calvignac-Spencer, S., Merkel, K., Kutzner, N., Kühl, H., Boesch, C., Kappeler, P.M., Metzger, S., Schubert, G., Leendertz, F.H., 2013. Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. Mol. Ecol. 22, 915–924.

Carter, R., 2003. Speculations on the origins of Plasmodium vivax malaria. Trends Parasitol 19, 214–219. doi:10.1016/s1471-4922(03)00070-9

Carter, R., Mendis, K.N., 2002. Evolutionary and historical aspects of the burden of malaria. Clin Microbiol Rev 15, 564–594.

CDC, 2012. Anopheles mosquitoes [WWW Document]. URL http://www.cdc.gov/malaria/about/biology/mosquitoes/

Coatney, G.R., 1968. Simian malarias in man: facts, implications, and predictions. Am J Trop Med Hyg 17, 147–155.

Coetzee, M., 2004. Distribution of the African malaria vectors of the Anopheles gambiae complex. Am J Trop Med Hyg 70, 103–104.

Collins, W.E., Jeffery, G.M., 2007. Plasmodium malariae: parasite and disease. Clin Microbiol Rev 20, 579–592. doi:10.1128/CMR.00027-07

Collins, W.E., Jeffery, G.M., 2005. Plasmodium ovale: parasite and disease. Clin Microbiol Rev 18, 570–581. doi:10.1128/CMR.18.3.570-581.2005

Collins, W.E., Skinner, J.C., Pappaioanou, M., Broderson, J.R., Mehaffey, P., 1986. The sporogonic cycle of Plasmodium reichenowi. J. Parasitol. 72, 292–8.

Contacos, P.G., Coatney, G.R., Orihel, T.C., Collins, W.E., Chin, W., Jeter, M.H., 1970. Transmission of Plasmodium schwetzi from the chimpanzee to man by mosquito bite. Am J Trop Med Hyg 19, 190–195.

Conway, D.J., Fanello, C., Lloyd, J.M., Al-Joubori, B.M., Baloch, A.H., Somanath, S.D., Roper, C., Oduola, A.M., Mulder, B., Povoa, M.M., Singh, B., Thomas, A.W., 2000. Origin of Plasmodium falciparum malaria is traced by mitochondrial DNA. Mol Biochem Parasitol 111, 163–171.

Culleton, R.L., Mita, T., Ndounga, M., Unger, H., Cravo, P. V, Paganotti, G.M., Takahashi, N., Kaneko, A., Eto, H., Tinto, H., Karema, C., D'Alessandro, U., do Rosario, V., Kobayakawa, T., Ntoumi, F., Carter, R., Tanabe, K., 2008. Failure to detect Plasmodium vivax in West and Central Africa by PCR species typing. Malar J 7, 174. doi:10.1186/1475-2875-7-174

Davies, C.R., Ayres, J.M., Dye, C., Deane, L.M., 1991. Malaria infection rate of Amazonian primates increases with body weight and group size. Funct. Ecol. 5, 655–662.

De Monbrison, F., Angei, C., Staal, A., Kaiser, K., Picot, S., 2003. Simultaneous identification of the four human Plasmodium species and quantification of Plasmodium DNA load in human blood by real-time polymerase chain reaction. Trans R Soc Trop Med Hyg 97, 387–390.

De Nys, H.M., Calvignac-Spencer, S., Boesch, C., Dorny, P., Wittig, R.M., Mundry, R., Leendertz, F.H., 2014. Malaria parasite detection increases during pregnancy in wild chimpanzees. Malar J 13, 413. doi:10.1186/1475-2875-13-413

De Nys, H.M., Calvignac-Spencer, S., Thiesen, U., Boesch, C., Wittig, R.M., Mundry, R., Leendertz, F.H., 2013. Age-related effects on malaria parasite infection in wild chimpanzees. Biol. Lett. 9, 20121160. doi:10.1098/rsbl.2012.1160

Délicat-Loembet, L., Rougeron, V., Ollomo, B., Arnathau, C., Roche, B., Elguero, E., Moukodoum, N.D., Okougha, A.-P., Mve Ondo, B., Boundenga, L., Houzé, S., Galan, M., Nkoghé, D., Leroy, E.M., Durand, P., Paupy, C., Renaud, F., Prugnolle, F., 2015. No Evidence for Ape Plasmodium Infections in Humans in Gabon. PLoS One 10, e0126933. doi:10.1371/journal.pone.0126933

Desai, M., ter Kuile, F.O., Nosten, F., McGready, R., Asamoa, K., Brabin, B., Newman, R.D., 2007. Epidemiology and burden of malaria in pregnancy. Lancet Infect. Dis. 7, 93–104. doi:10.1016/s1473-3099(07)70021-x

Dobson, A.J., 2002. An introduction to generalized linear models. Chapman et Hall/CRC, Boca Raton, USA.

Doolan, D.L., Dobaño, C., Baird, J.K., 2009. Acquired Immunity to Malaria. Clin. Microbiol. Rev. 22, 13–36.

Duval, L., Ariey, F., 2012. Ape Plasmodium parasites as a source of human outbreaks. Clin Microbiol Infect 18(6), 528-532. doi: 10.1111/j.1469-0691.2012.03825.x.

Duval, L., Fourment, M., Nerrienet, E., Rousset, D., Sadeuh, S.A., Goodman, S.M., Andriaholinirina, N. V, Randrianarivelojosia, M., Paul, R.E., Robert, V., Ayala, F.J., Ariey, F., 2010. African apes as reservoirs of Plasmodium falciparum and the origin and diversification of the Laverania subgenus. Proc Natl Acad Sci U S A 107, 10561–10566. doi:10.1073/pnas.1005435107

Duval, L., Nerrienet, E., Rousset, D., Sadeuh Mba, S.A., Houze, S., Fourment, M., Le Bras, J., Robert, V., Ariey, F., 2009. Chimpanzee malaria parasites related to Plasmodium ovale in Africa. PLoS One 4, e5520. doi:10.1371/journal.pone.0005520

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32, 1792–1797. doi:10.1093/nar/gkh340

Efunshile, A.M., Olawale, T., Stensvold, C.R., Kurtzhals, J.A., Konig, B., 2015. Epidemiological study of the association between malaria and helminth infections in Nigeria. Am J Trop Med Hyg 92, 578–582. doi:10.4269/ajtmh.14-0548

Ehlers, B., Spiess, K., Leendertz, F., Peeters, M., Boesch, C., Gatherer, D., McGeoch, D.J., 2010. Lymphocryptovirus phylogeny and the origins of Epstein-Barr virus. J. Gen. Virol. 91, 630–42. doi:10.1099/vir.0.017251-0

Ekvall, H., Arese, P., Turrini, F., Ayi, K., Mannu, F., Premji, Z., Bjorkman, A.,. Acute haemolysis in childhood falciparum malaria. Trans. R. Soc. Trop. Med. Hyg. 95, 611–7.

Escalante, A.A., Ayala, F.J., 1994. Phylogeny of the malarial genus Plasmodium, derived from rRNA gene sequences. Proc. Natl. Acad. Sci. U. S. A. 91, 11373–7.

Escalante, A.A., Barrio, E., Ayala, F.J., 1995. Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. Mol Biol Evol 12, 616–626.

Escalante, A.A., Cornejo, O.E., Freeland, D.E., Poe, A.C., Durrego, E., Collins, W.E., Lal, A.A., 2005. A monkey's tale: the origin of Plasmodium vivax as a human malaria parasite. Proc Natl Acad Sci U S A 102, 1980–1985. doi:10.1073/pnas.0409652102

Escalante, A.A., Freeland, D.E., Collins, W.E., Lal, A.A., 1998. The evolution of primate malaria parasites based on the gene encoding cytochrome b from the linear mitochondrial genome. Proc. Natl. Acad. Sci. 95, 8124–8129. doi:10.1073/pnas.95.14.8124

Etienne, L., Nerrienet, E., LeBreton, M., Bibila, G.T., Foupouapouognigni, Y., Rousset, D., Nana, A., Djoko, C.F., Tamoufe, U., Aghokeng, A.F., Mpoudi-Ngole, E., Delaporte, E., Peeters, M., Wolfe, N.D., Ayouba, A., 2011. Characterization of a new simian immunodeficiency virus strain in a naturally infected Pan troglodytes troglodytes chimpanzee with AIDS related symptoms. Retrovirology 8, 4. doi:10.1186/1742-4690-8-4

Fahy, G.E., Richards, M., Riedel, J., Hublin, J.J., Boesch, C., 2013. Stable isotope evidence of meat eating and hunting specialization in adult male chimpanzees. Proc Natl Acad Sci U S A 110, 5829–5833. doi:10.1073/pnas.1221991110

Faure, E., 2014. Malarial pathocoenosis: beneficial and deleterious interactions between malaria and other human diseases. Front Physiol 5, 441. doi:10.3389/fphys.2014.00441

Fernandez-Nino, J.A., Idrovo, A.J., Cucunuba, Z.M., Reyes-Harker, P., Guerra, A.P., Moncada, L.I., Lopez, M.C., Barrera, S.M., Cortes, L.J., Olivera, M., Nicholls, R.S., 2012. Paradoxical associations between soil-transmitted helminths and Plasmodium falciparum infection. Trans. R. Soc. Trop. Med. Hyg. 106, 701–708.

Field, A.P., 2005. Discovering statistics using SPSS : (and sex, drugs and rock "n" roll), 2nd ed, ISM introducing statistical methods. Sage Publications, London ; Thousand Oaks, Calif.

Fievet, N., Cot, M., Chougnet, C., Maubert, B., Bickii, J., Dubois, B., Le Hesran, J.Y., Frobert, Y., Migot, F., Romain, F., 1995. Malaria and pregnancy in Cameroonian primigravidae: humoral and cellular immune responses to Plasmodium falciparum blood-stage antigens. Am. J. Trop. Med. Hyg. 53, 612–7.

Flahou, B., Modrý, D., Pomajbíková, K., Petrželková, K.J., Smet, A., Ducatelle, R., Pasmans, F., Sá, R.M., Todd, A., Hashimoto, C., Mulama, M., Kiang, J., Rossi, M., Haesebrouck, F., 2014. Diversity of zoonotic enterohepatic Helicobacter species and detection of a putative novel gastric Helicobacter species in wild and wild-born captive chimpanzees and western lowland gorillas. Vet. Microbiol. doi:10.1016/j.vetmic.2014.08.032

Forstmeier, W., Schielzeth, H., 2011. Cryptic multiple hypotheses testing in linear models: overestimated effect sizes and the winner's curse. Behav Ecol Sociobiol 65, 47–55. doi:10.1007/s00265-010-1038-5

Frantzen, M.A., Silk, J.B., Ferguson, J.W., Wayne, R.K., Kohn, M.H., 1998. Empirical evaluation of preservation methods for faecal DNA. Mol. Ecol. 7, 1423–8.

Fujita, S., Kageyama, T., 2007. Polymerase chain reaction detection of Clostridium perfringens in feces from captive and wild chimpanzees, Pan troglodytes. J. Med. Primatol. 36, 25–32. doi:10.1111/j.1600-0684.2006.00191.x

Furtbauer, I., Mundry, R., Heistermann, M., Schulke, O., Ostner, J., 2011. You mate, I mate: macaque females synchronize sex not cycles. PLoS One 6, e26144. doi:10.1371/journal.pone.0026144

Garnham, P.C., Lainson, R., Gunders, A.E., 1956. Some observations on malaria parasites in a chimpanzee, with particular reference to the persistence of Plasmodium reichenowi and Plasmodium vivax. Ann Soc Belg Med Trop 36, 811–821.

Gautret, P., Legros, F., Koulmann, P., Rodier, M.H., Jacquemin, J.L., 2001. Imported Plasmodium vivax malaria in France: Geographical origin and report of an atypical case acquired in Central or Western Africa, in: Acta Tropica. pp. 177–181. doi:10.1016/S0001-706X(00)00181-9

Gillespie, T.R., Nunn, C.L., Leendertz, F.H., 2008. Integrative approaches to the study of primate infectious disease: implications for biodiversity conservation and global health. Am. J. Phys. Anthropol. Suppl 47, 53–69. doi:10.1002/ajpa.20949

Gogarten, J.F., Akoua-Koffi, C., Calvignac-Spencer, S., Leendertz, S.A., Weiss, S., Couacy-Hymann, E., Kone, I., Peeters, M., Wittig, R.M., Boesch, C., Hahn, B.H., Leendertz, F.H., 2014. The ecology of primate retroviruses - an assessment of 12 years of retroviral studies in the Tai national park area, Cote dIvoire. Virology 460-461, 147–153. doi:10.1016/j.virol.2014.05.012

Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol Biol Evol 27, 221–224. doi:10.1093/molbev/msp259

Graham, A.L., 2008. Ecological rules governing helminth-microparasite coinfection. Proc Natl Acad Sci U S A 105, 566–570. doi:10.1073/pnas.0707221105

Griffiths, E.C., Pedersen, A.B., Fenton, A., Petchey, O.L., 2014. Analysis of a summary network of co-infection in humans reveals that parasites interact most via shared resources. Proc Biol Sci 281, 20132286. doi:10.1098/rspb.2013.2286

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol 59, 307–321. doi:10.1093/sysbio/syq010

Guyatt, H.L., Snow, R.W., 2001. Malaria in pregnancy as an indirect cause of infant mortality in sub-Saharan Africa. Trans. R. Soc. Trop. Med. Hyg. 95, 569–76.

Harvala, H., Sharp, C.P., Ngole, E.M., Delaporte, E., Peeters, M., Simmonds, P., 2011. Detection and genetic characterization of enteroviruses circulating among wild populations of chimpanzees in Cameroon: relationship with human and simian enteroviruses. J Virol 85, 4480–4486. doi:10.1128/JVI.02285-10

Harvala, H., Van Nguyen, D., McIntyre, C., Ahuka-Mundeke, S., Mpoudi Ngole, E., Delaporte, E., Peeters, M., Simmonds, P., 2013. Co-circulation of enteroviruses between apes and humans. J. Gen. Virol. doi:10.1099/vir.0.059048-0

Hayakawa, T., Arisue, N., Udono, T., Hirai, H., Sattabongkot, J., Toyama, T., Tsuboi, T., Horii, T., Tanabe, K., 2009. Identification of Plasmodium malariae, a human malaria parasite, in imported chimpanzees. PLoS One 4, e7412. doi:10.1371/journal.pone.0007412

Hayakawa, T., Culleton, R., Otani, H., Horii, T., Tanabe, K., 2008. Big bang in the evolution of extant malaria parasites. Mol Biol Evol 25, 2233–2239. doi:10.1093/molbev/msn171

Head, J.S., Boesch, C., Robbins, M.M., Rabanal, L.I., Makaga, L., Kühl, H.S., 2013. Effective sociodemographic population assessment of elusive species in ecology and conservation management. Ecol. Evol. 3, 2903–2916. doi:10.1002/ece3.670

Herbert, A., Boundenga, L., Meyer, A., Moukodoum, D.N., Okouga, A.P., Arnathau, C., Durand, P., Magnus, J., Ngoubangoye, B., Willaume, E., Ba, C.T., Rougeron, V., Renaud, F., Ollomo, B., Prugnolle, F., 2015. Malaria-like symptoms associated with a natural Plasmodium reichenowi infection in a chimpanzee. Malar. J. 14, 220. doi:10.1186/s12936-015-0743-y

Hill, K., Boesch, C., Goodall, J., Pusey, A., Williams, J., Wrangham, R., 2001. Mortality rates among wild chimpanzees. J Hum Evol 40, 437–450. doi:10.1006/jhev.2001.0469

Hill, A. V, Allsopp, C.E., Kwiatkowski, D., Anstey, N.M., Twumasi, P., Rowe, P.A., Bennett, S., Brewster, D., McMichael, A.J., Greenwood, B.M., 1991. Common west African HLA antigens are associated with protection from severe malaria. Nature 352, 595–600. doi:10.1038/352595a0

Hirakawa, S., Omori, S., Sato, Y., Isobe, T., Yukawa, M., Murata, K., 2007. Complete nucleotide sequences of the mitochondrial genome of Leucocytozoon sabrazesi. Unpublished. NCBI Direct Submission.

Hofreiter, M., Kreuz, E., Eriksson, J., Schubert, G., Hohmann, G., 2010. Vertebrate DNA in fecal samples from bonobos and gorillas: evidence for meat consumption or artefact? PLoS One 5, e9419. doi:10.1371/journal.pone.0009419

Hughes, A.L., Verra, F., 2010. Malaria parasite sequences from chimpanzee support the co-speciation hypothesis for the origin of virulent human malaria (Plasmodium falciparum). Mol Phylogenet Evol 57, 135–143. doi:10.1016/j.ympev.2010.06.004

Jeffares, D.C., Pain, A., Berry, A., Cox, A. V, Stalker, J., Ingle, C.E., Thomas, A., Quail, M.A., Siebenthall, K., Uhlemann, A.C., Kyes, S., Krishna, S., Newbold, C., Dermitzakis, E.T.,

Berriman, M., 2007. Genome variation and evolution of the malaria parasite Plasmodium falciparum. Nat Genet 39, 120–125. doi:10.1038/ng1931

Jensen, S.A., Mundry, R., Nunn, C.L., Boesch, C., Leendertz, F.H., 2009. Non-invasive body temperature measurement of wild chimpanzees using fecal temperature decline. J Wildl Dis 45, 542–546.

Jirku, M., Pomajbikova, K., Petrzelkova, K.J., Huzova, Z., Modry, D., Lukes, J., 2012. Detection of Plasmodium spp. in human feces. Emerg Infect Dis 18, 634–636. doi:10.3201/eid1804.110984

Jongwutiwes, S., Putaporntip, C., Iwasaki, T., Ferreira, M.U., Kanbara, H., Hughes, A.L., 2005. Mitochondrial genome sequences support ancient population expansion in Plasmodium vivax. Mol Biol Evol 22, 1733–1739. doi:10.1093/molbev/msi168

Joy, D.A., Feng, X., Mu, J., Furuya, T., Chotivanich, K., Krettli, A.U., Ho, M., Wang, A., White, N.J., Suh, E., Beerli, P., Su, X.Z., 2003. Early origin and recent expansion of Plasmodium falciparum. Science (80-.). 300, 318–321. doi:10.1126/science.1081449

Junglen, S., Kurth, A., Kuehl, H., Quan, P.L., Ellerbrok, H., Pauli, G., Nitsche, A., Nunn, C., Rich, S.M., Lipkin, W.I., Briese, T., Leendertz, F.H., 2009. Examining landscape factors influencing relative distribution of mosquito genera and frequency of virus infection. Ecohealth 6, 239–249. doi:10.1007/s10393-009-0260-y

Kaiser, M., Lowa, A., Ulrich, M., Ellerbrok, h., Goffe, A.S., Blasse, A., Zommers, Z., Couacy-Hymann, E., Babweteera, F., Zuberbuhler, K., Metzger, S., Geidel, S., Boesch, C., Gillepsie, T., Leendertz, F., 2010. Wild Chimpanzees Infected with 5 Plasmodium Species. Emerg Infect Dis. doi:10.3201/eid1612.100424

Kawai, S., Sato, M., Kato-Hayashi, N., Kishi, H., Huffman, M.A., Maeno, Y., Culleton, R., Nakazawa, S., 2014. Detection of Plasmodium knowlesi DNA in the urine and faeces of a Japanese macaque (Macaca fuscata) over the course of an experimentally induced infection. Malar. J. 13, 373. doi:10.1186/1475-2875-13-373

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. doi:10.1093/bioinformatics/bts199

Keele, B.F., Jones, J.H., Terio, K.A., Estes, J.D., Rudicell, R.S., Wilson, M.L., Li, Y., Learn, G.H., Beasley, T.M., Schumacher-Stankey, J., Wroblewski, E., Mosser, A., Raphael, J., Kamenya, S., Lonsdorf, E. V, Travis, D.A., Mlengeya, T., Kinsel, M.J., Else, J.G., Silvestri, G., Goodall, J., Sharp, P.M., Shaw, G.M., Pusey, A.E., Hahn, B.H., 2009. Increased mortality and AIDS-like immunopathology in wild chimpanzees infected with SIVcpz. Nature 460, 515–519. doi:10.1038/nature08200

Keele, B.F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M.L., Bibollet-Ruche, F., Chen, Y., Wain, L. V, Liegeois, F., Loul, S., Ngole, E.M., Bienvenue, Y., Delaporte, E.,

Brookfield, J.F., Sharp, P.M., Shaw, G.M., Peeters, M., Hahn, B.H., 2006. Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. Science (80-.). 313, 523–526. doi:10.1126/science.1126531

Keita, A.K., Socolovschi, C., Ahuka-Mundeke, S., Ratmanov, P., Butel, C., Ayouba, A., Inogwabini, B.-I., Muyembe-Tamfum, J.-J., Mpoudi-Ngole, E., Delaporte, E., Peeters, M., Fenollar, F., Raoult, D., 2013. Molecular evidence for the presence of Rickettsia Felis in the feces of wild-living African apes. PLoS One 8, e54679. doi:10.1371/journal.pone.0054679

Kelly-Hope, L.A., Diggle, P.J., Rowlingson, B.S., Gyapong, J.O., Kyelem, D., Coleman, M., Thomson, M.C., Obsomer, V., Lindsay, S.W., Hemingway, J., Molyneux, D.H., 2006. Short communication: Negative spatial association between lymphatic filariasis and malaria in West Africa. Trop Med Int Heal. 11, 129–135. doi:10.1111/j.1365-3156.2005.01558.x

Kondgen, S., Schenk, S., Pauli, G., Boesch, C., Leendertz, F.H., 2010. Noninvasive monitoring of respiratory viruses in wild chimpanzees. Ecohealth 7, 332–341. doi:10.1007/s10393-010-0340-z

Koops, K., McGrew, W.C., Matsuzawa, T., Knapp, L.A., 2012. Terrestrial nest-building by wild chimpanzees (Pan troglodytes): implications for the tree-to-ground sleep transition in early hominins. Am J Phys Anthr. 148, 351–361. doi:10.1002/ajpa.22056

Kotepui, M., Piwkham, D., PhunPhuech, B., Phiwklam, N., Chupeerach, C., Duangmano, S., 2015. Effects of malaria parasite density on blood cell parameters. PLoS One 10, e0121057. doi:10.1371/journal.pone.0121057

Krebs, B.L., Anderson, T.K., Goldberg, T.L., Hamer, G.L., Kitron, U.D., Newman, C.M., Ruiz, M.O., Walker, E.D., Brawn, J.D., 2014. Host group formation decreases exposure to vector-borne disease: a field experiment in a “hotspot” of West Nile virus transmission. Proc Biol Sci 281, 20141586. doi:10.1098/rspb.2014.1586

Krief, S., Escalante, A.A., Pacheco, M.A., Mugisha, L., Andre, C., Halbwax, M., Fischer, A., Krief, J.M., Kasenene, J.M., Crandfield, M., Cornejo, O.E., Chavatte, J.M., Lin, C., Letourneur, F., Gruner, A.C., McCutchan, T.F., Renia, L., Snounou, G., 2010. On the diversity of malaria parasites in African apes and the origin of Plasmodium falciparum from Bonobos. PLoS Pathog 6, e1000765. doi:10.1371/journal.ppat.1000765

Krief, S., Levrero, F., Krief, J.-M., Thanapongpichat, S., Imwong, M., Snounou, G., Kasenene, J.M., Cibot, M., Gantier, J.-C., 2012. Investigations on anopheline mosquitoes close to the nest sites of chimpanzees subject to malaria infection in Ugandan Highlands. Malar. J. 11, 116. doi:10.1186/1475-2875-11-116

Kwiatkowski, D.P., 2005. How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet 77, 171–192. doi:10.1086/432519

Lal, A.A., Goldman, I.F., 1991. Circumsporozoite protein gene from Plasmodium reichenowi, a chimpanzee malaria parasite evolutionarily related to the human malaria parasite Plasmodium falciparum. J Biol Chem 266, 6686–6689.

Lee, K.S., Divis, P.C., Zakaria, S.K., Matusop, A., Julin, R.A., Conway, D.J., Cox-Singh, J., Singh, B., 2011. Plasmodium knowlesi: reservoir hosts and tracking the emergence in humans and macaques. PLoS Pathog 7, e1002015. doi:10.1371/journal.ppat.1002015

Leendertz, F.H., Boesch, C., Ellerbrok, H., Rietschel, W., Couacy-Hymann, E., Pauli, G., 2004. Non-invasive testing reveals a high prevalence of simian T-lymphotropic virus type 1 antibodies in wild adult chimpanzees of the Tai National Park, Cote d'Ivoire. J Gen Virol 85, 3305–3312. doi:10.1099/vir.0.80052-0

Leendertz, F.H., Lankester, F., Guislain, P., Néel, C., Drori, O., Dupain, J., Speede, S., Reed, P., Wolfe, N., Loul, S., Mpoudi-Ngole, E., Peeters, M., Boesch, C., Pauli, G., Ellerbrok, H., Leroy, E.M., 2006. Anthrax in Western and Central African great apes. Am. J. Primatol. 68, 928–933.

Leendertz, F.H., Pauli, G., Maetz-Rensing, K., Boardman, W., Nunn, C., Ellerbrok, H., Jensen, S.A., Junglen, S., Christophe, B., 2006. Pathogens as drivers of population declines: The importance of systematic monitoring in great apes and other threatened mammals. Biol. Conserv. 131, 325–337. doi:10.1016/j.biocon.2006.05.002

Leke, R.F.G., Bioga, J.D., Zhou, J., Fouda, G.G., Leke, R.J.I., Tchinda, V., Megnekou, R., Fogako, J., Sama, G., Gwanmesia, P., Bomback, G., Nama, C., Diouf, A., Bobbili, N., Taylor, D.W., 2010. Longitudinal studies of Plasmodium falciparum malaria in pregnant women living in a rural Cameroonian village with high perennial transmission. Am. J. Trop. Med. Hyg. 83, 996–1004. doi:10.4269/ajtmh.2010.10-0249

Li, L., Kapoor, A., Slikas, B., Bamidele, O.S., Wang, C., Shaukat, S., Masroor, M.A., Wilson, M.L., Ndjango, J.-B.N., Peeters, M., Gross-Camp, N.D., Muller, M.N., Hahn, B.H., Wolfe, N.D., Triki, H., Bartkus, J., Zaidi, S.Z., Delwart, E., 2010. Multiple diverse circoviruses infect farm animals and are commonly found in human and chimpanzee feces. J. Virol. 84, 1674–82. doi:10.1128/JVI.02109-09

Li, Y., Ndjango, J.-B., Learn, G.H., Ramirez, M.A., Keele, B.F., Bibollet-Ruche, F., Liu, W., Easlick, J.L., Decker, J.M., Rudicell, R.S., Inogwabini, B.-I., Ahuka-Mundeke, S., Leendertz, F.H., Reynolds, V., Muller, M.N., Chancellor, R.L., Rundus, A.S., Simmons, N., Worobey, M., Shaw, G.M., Peeters, M., Sharp, P.M., Hahn, B.H., 2012. Eastern chimpanzees, but not bonobos, represent a simian immunodeficiency virus reservoir. J. Virol. 86, 10776–91. doi:10.1128/JVI.01498-12

Lindsay, S., Ansell, J., Selman, C., Cox, V., Hamilton, K., Walraven, G., 2000. Effect of pregnancy on exposure to malaria mosquitoes. Lancet 355, 1972. doi:10.1016/S0140-6736(00)02334-5

Liu, W., Li, Y., Learn, G.H., Rudicell, R.S., Robertson, J.D., Keele, B.F., Ndjango, J.B., Sanz, C.M., Morgan, D.B., Locatelli, S., Gonder, M.K., Kranzusch, P.J., Walsh, P.D., Delaporte, E., Mpoudi-Ngole, E., Georgiev, A. V, Muller, M.N., Shaw, G.M., Peeters, M., Sharp, P.M.,

Rayner, J.C., Hahn, B.H., 2010. Origin of the human malaria parasite Plasmodium falciparum in gorillas. Nature 467, 420–425. doi:10.1038/nature09442

Liu, W., Li, Y., Shaw, K.S., Learn, G.H., Plenderleith, L.J., Malenke, J.A., Sundararaman, S.A., Ramirez, M.A., Crystal, P.A., Smith, A.G., Bibollet-Ruche, F., Ayouba, A., Locatelli, S., Esteban, A., Mouacha, F., Guichet, E., Butel, C., Ahuka-Mundeke, S., Inogwabini, B.-I., Ndjango, J.-B.N., Speede, S., Sanz, C.M., Morgan, D.B., Gonder, M.K., Kranzusch, P.J., Walsh, P.D., Georgiev, A. V, Muller, M.N., Piel, A.K., Stewart, F.A., Wilson, M.L., Pusey, A.E., Cui, L., Wang, Z., Färnert, A., Sutherland, C.J., Nolder, D., Hart, J.A., Hart, T.B., Bertolani, P., Gillis, A., LeBreton, M., Tafon, B., Kiyang, J., Djoko, C.F., Schneider, B.S., Wolfe, N.D., Mpoudi-Ngole, E., Delaporte, E., Carter, R., Culleton, R.L., Shaw, G.M., Rayner, J.C., Peeters, M., Hahn, B.H., Sharp, P.M., 2014. African origin of the malaria parasite Plasmodium vivax. Nat. Commun. 5, 3346. doi:10.1038/ncomms4346

Liu, W., Worobey, M., Li, Y., Keele, B.F., Bibollet-Ruche, F., Guo, Y., Goepfert, P.A., Santiago, M.L., Ndjango, J.-B.N., Neel, C., Clifford, S.L., Sanz, C., Kamenya, S., Wilson, M.L., Pusey, A.E., Gross-Camp, N., Boesch, C., Smith, V., Zamma, K., Huffman, M.A., Mitani, J.C., Watts, D.P., Peeters, M., Shaw, G.M., Switzer, W.M., Sharp, P.M., Hahn, B.H., 2008. Molecular ecology and natural history of simian foamy virus infection in wild-living chimpanzees. PLoS Pathog. 4, e1000097. doi:10.1371/journal.ppat.1000097

Lupi, P., 2003. How immune mechanisms are affected by pregnancy. Vaccine 21(24), 3352-3357. doi:10.1016/S0264-410X(03)00331-1

MacFie, T.S., Nerrienet, E., Bontrop, R.E., Mundy, N.I., 2009. The action of falciparum malaria on the human and chimpanzee genomes compared: absence of evidence for a genomic signature of malaria at HBB and G6PD in three subspecies of chimpanzee. Infect. Genet. Evol. 9, 1248–52. doi:10.1016/j.meegid.2009.06.025

Makuwa, M., Souquière, S., Clifford, S.L., Mouinga-Ondeme, A., Bawe-Johnson, M., Wickings, E.J., Latour, S., Simon, F., Roques, P., 2005. Identification of hepatitis B virus genome in faecal sample from wild living chimpanzee (Pan troglodytes troglodytes) in Gabon. J. Clin. Virol. 34, S83–S88. doi:10.1016/S1386-6532(05)80016-2

Mapua, M.I., Qablan, M.A., Pomajbíková, K., Petrželková, K.J., Hůzová, Z., Rádrová, J., Votýpka, J., Todd, A., Jirků, M., Leendertz, F.H., Lukeš, J., Neel, C., Modrý, D., 2015. Ecology of malaria infections in western lowland gorillas inhabiting Dzanga Sangha Protected Areas, Central African Republic. Parasitology 142(7), 890-900. doi: 10.1017/S0031182015000086

Martin, M.J., Rayner, J.C., Gagneux, P., Barnwell, J.W., Varki, A., 2005. Evolution of human-chimpanzee differences in malaria susceptibility: relationship to human genetic loss of N-glycolylneuraminic acid. Proc Natl Acad Sci U S A 102, 12819–12824. doi:10.1073/pnas.0503819102

Martinsen, E.S., Perkins, S.L., Schall, J.J., 2008. A three-genome phylogeny of malaria parasites (Plasmodium and closely related genera): evolution of life-history traits and host switches. Mol Phylogenet Evol 47 (1), 261-273. doi: 10.1016/j.ympev.2007.11.012

Mayxay, M., Pukrittayakamee, S., Newton, P.N., White, N.J., 2004. Mixed-species malaria infections in humans. Trends Parasitol 20, 233–240. doi:10.1016/j.pt.2004.03.006

McGregor, I.A., 1984. Epidemiology, malaria and pregnancy. Am J Trop Med Hyg 33, 517–525.

Mendis, K., Sina, B.J., Marchesini, P., Carter, R., 2001. The neglected burden of Plasmodium vivax malaria. Am J Trop Med Hyg 64, 97–106.

Mharakurwa, S., Simoloka, C., Thuma, P.E., Shiff, C.J., Sullivan, D.J., 2006. PCR detection of Plasmodium falciparum in human urine and saliva samples. Malar J 5, 103. doi:10.1186/1475-2875-5-103

Minhos, T., Nixon, E., Sousa, C., Vicente, L.M., da Silva, M.F., Sa, R., Bruford, M.W., 2013. Genetic evidence for spatio-temporal changes in the dispersal patterns of two sympatric African colobine monkeys. Am J Phys Anthr. 150, 464–474. doi:10.1002/ajpa.22223

Mooring, M.S., Hart, B.L., 1992. Animal Grouping for Protection from Parasites: Selfish Herd and Encounter-Dilution Effects. Behaviour 123, 173–193. doi:10.2307/4535069

Morgan, B.J., 2007. Group size, density and biomass of large mammals in the Réserve de Faune du Petit Loango, Gabon. Afr. J. Ecol. 45, 508–518. doi:10.1111/j.1365-2028.2007.00761.x

Mu, J., Joy, D.A., Duan, J., Huang, Y., Carlton, J., Walker, J., Barnwell, J., Beerli, P., Charleston, M.A., Pybus, O.G., Su, X.Z., 2005. Host switch leads to emergence of Plasmodium vivax malaria in humans. Mol Biol Evol 22, 1686–1693. doi:10.1093/molbev/msi160

Mühlberger, N., Jelinek, T., Gascon, J., Probst, M., Zoller, T., Schunk, M., Beran, J., Gjørup, I., Behrens, R.H., Clerinx, J., Björkman, A., McWhinney, P., Matteelli, A., Lopez-Velez, R., Bisoffi, Z., Hellgren, U., Puente, S., Schmid, M.L., Myrvang, B., Holthoff-Stich, M.L., Laferl, H., Hatz, C., Kollaritsch, H., Kapaun, A., Knobloch, J., Iversen, J., Kotlowski, A., Malvy, D.J.M., Kern, P., Fry, G., Siikamaki, H., Schulze, M.H., Soula, G., Paul, M., Gómez i Prat, J., Lehmann, V., Bouchaud, O., da Cunha, S., Atouguia, J., Boecken, G., 2004. Epidemiology and clinical features of vivax malaria imported to Europe: sentinel surveillance data from TropNetEurop. Malar. J. 3, 5. doi:10.1186/1475-2875-3-5

Neel, C., Etienne, L., Li, Y., Takehisa, J., Rudicell, R.S., Bass, I.N., Moudindo, J., Mebenga, A., Esteban, A., Van Heuverswyn, F., Liegeois, F., Kranzusch, P.J., Walsh, P.D., Sanz, C.M., Morgan, D.B., Ndjango, J.-B.N., Plantier, J.-C., Locatelli, S., Gonder, M.K., Leendertz, F.H., Boesch, C., Todd, A., Delaporte, E., Mpoudi-Ngole, E., Hahn, B.H., Peeters, M., 2010. Molecular epidemiology of simian immunodeficiency virus infection in wild-living gorillas. J. Virol. 84, 1464–76. doi:10.1128/JVI.02129-09

Nunn, C., Eckhard, W.H., 2005. Malaria infection and host behavior: a comparative study of Neotropical primates . Behav. Ecol. Sociobiol. 59, 30–37. doi:10.1007/s00265-005-0005-z

Nwakanma, D.C., Gomez-Escobar, N., Walther, M., Crozier, S., Dubovsky, F., Malkin, E., Locke, E., Conway, D.J., 2009. Quantitative detection of Plasmodium falciparum DNA in saliva, blood, and urine. J Infect Dis 199, 1567–1574. doi:10.1086/598856

O'Rorke, R., Lavery, S., Jeffs, A., 2012. PCR enrichment techniques to identify the diet of predators. Mol Ecol Resour 12, 5–17. doi:10.1111/j.1755-0998.2011.03091.x

Okenu, D.M., Thomas, A.W., Conway, D.J., 2000. Allelic lineages of the merozoite surface protein 3 gene in Plasmodium reichenowi and Plasmodium falciparum. Mol. Biochem. Parasitol. 109, 185–8.

Ollomo, B., Durand, P., Prugnolle, F., Douzery, E., Arnathau, C., Nkoghe, D., Leroy, E., Renaud, F., 2009. A new malaria agent in African hominids. PLoS Pathog 5, e1000446. doi:10.1371/journal.ppat.1000446

Omori, S., Sato, Y., Isobe, T., Yukawa, M., Murata, K., 2007. Complete nucleotide sequences of the mitochondrial genomes of two avian malaria protozoa, Plasmodium gallinaceum and Plasmodium juxtanucleare. Parasitol Res 100, 661–664. doi:10.1007/s00436-006-0333-6

Oni, G.A., Oguntibeju, O.O., 2006. Relationships between the Malaria Parasite Density and Children Anaemia: Brief Communication. Int. J. Pathol. 4.

Pacheco, M.A., Cranfield, M., Cameron, K., Escalante, A.A., 2013. Malarial parasite diversity in chimpanzees: the value of comparative approaches to ascertain the evolution of Plasmodium falciparum antigens. Malar. J. 12, 328. doi:10.1186/1475-2875-12-328

Patnaik, P., Jere, C.S., Miller, W.C., Hoffman, I.F., Wirima, J., Pendame, R., Meshnick, S.R., Taylor, T.E., Molyneux, M.E., Kublin, J.G., 2005. Effects of HIV-1 serostatus, HIV-1 RNA concentration, and CD4 cell count on the incidence of malaria infection in a cohort of adults in rural Malawi. J Infect Dis 192, 984–991. doi:10.1086/432730

Paula, D.P., Linard, B., Andow, D.A., Sujii, E.R., Pires, C.S.S., Vogler, A.P., 2015. Detection and decay rates of prey and prey symbionts in the gut of a predator through metagenomics. Mol Ecol Resour n/a–n/a. doi:10.1111/1755-0998.12364

Paupy, C., Makanga, B., Ollomo, B., Rahola, N., Durand, P., Magnus, J., Willaume, E., Renaud, F., Fontenille, D., Prugnolle, F., 2013. Anopheles moucheti and Anopheles vinckei Are Candidate Vectors of Ape Plasmodium Parasites, Including Plasmodium praefalciparum in Gabon. PLoS One 8, e57294. doi:10.1371/journal.pone.0057294

Pedersen, A.B., Fenton, A., 2007. Emphasizing the ecology in parasite community ecology. Trends Ecol Evol 22, 133–139. doi:10.1016/j.tree.2006.11.005

Perkins, S.L., 2008. Molecular systematics of the three mitochondrial protein-coding genes of malaria parasites: corroborative and new evidence for the origins of human malaria. Mitochondrial DNA 19, 471–478.

Perkins, S.L., 2006. Whole genome amplification and sequencing of mitochondrial DNA of saurian malaria parasites. Unpublished. NCBI. Direct submission.

Prugnolle, F., Durand, P., Neel, C., Ollomo, B., Ayala, F.J., Arnathau, C., Etienne, L., Mpoudi-Ngole, E., Nkoghe, D., Leroy, E., Delaporte, E., Peeters, M., Renaud, F., 2010. African great apes are natural hosts of multiple related malaria species, including Plasmodium falciparum. Proc Natl Acad Sci U S A 107, 1458–1463. doi:10.1073/pnas.0914440107

Prugnolle, F., Durand, P., Ollomo, B., Duval, L., Ariey, F., Arnathau, C., Gonzalez, J.P., Leroy, E., Renaud, F., 2011a. A fresh look at the origin of Plasmodium falciparum, the most malignant malaria agent. PLoS Pathog 7, e1001283. doi:10.1371/journal.ppat.1001283

Prugnolle, F., Ollomo, B., Durand, P., Yalcindag, E., Arnathau, C., Elguero, E., Berry, A., Pourrut, X., Gonzalez, J.P., Nkoghe, D., Akiana, J., Verrier, D., Leroy, E., Ayala, F.J., Renaud, F., 2011b. African monkeys are infected by Plasmodium falciparum nonhuman primate-specific strains. Proc Natl Acad Sci U S A. doi:10.1073/pnas.1109368108

Prugnolle, F., Rougeron, V., Becquart, P., Berry, A., Makanga, B., Rahola, N., Arnathau, C., Ngoubangoye, B., Menard, S., Willaume, E., Ayala, F.J., Fontenille, D., Ollomo, B., Durand, P., Paupy, C., Renaud, F., 2013. Diversity, host switching and evolution of Plasmodium vivax infecting African great apes. Proc. Natl. Acad. Sci. U. S. A. 110, 8123–8. doi:10.1073/pnas.1306004110

Putaporntip, C., Buppan, P., Jongwutiwes, S., 2011. Improved performance with saliva and urine as alternative DNA sources for malaria diagnosis by mitochondrial DNA-based PCR assays. Clin. Microbiol. Infect. 17, 1484–91. doi:10.1111/j.1469-0691.2011.03507.x

Quinn, G.P., Keough, M.J., 2002. Experimental Designs and Data Analysis for Biologists, in: Press, C.U. (Ed.), . Cambridge.

R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/. R Found. Stat. Comput. Vienna, Austria.

Rask, T.S., Hansen, D.A., Theander, T.G., Gorm Pedersen, A., Lavstsen, T., 2010. Plasmodium falciparum erythrocyte membrane protein 1 diversity in seven genomes--divide and conquer. PLoS Comput Biol 6. doi:10.1371/journal.pcbi.1000933

Rayner, J.C., Huber, C.S., Galinski, M.R., Barnwell, J.W., 2004. Rapid evolution of an erythrocyte invasion gene family: the Plasmodium reichenowi Reticulocyte Binding Like (RBL) genes. Mol. Biochem. Parasitol. 133, 287–96.

Rayner, J.C., Liu, W., Peeters, M., Sharp, P.M., Hahn, B.H., 2011. A plethora of Plasmodium species in wild apes: a source of human infection? Trends Parasitol 27, 222–229. doi:10.1016/j.pt.2011.01.006

Reichenow, E., 1920. Über das Vorkommen der Malariaparasiten des Menschen bei den Afrikanischen Menschenaffen. Cent. f.Bakt. I. Abt. Orig. 85, 207–216.

Rich, S.M., Leendertz, F.H., Xu, G., LeBreton, M., Djoko, C.F., Aminake, M.N., Takang, E.E., Diffo, J.L., Pike, B.L., Rosenthal, B.M., Formenty, P., Boesch, C., Ayala, F.J., Wolfe, N.D., 2009. The origin of malignant malaria. Proc Natl Acad Sci U S A 106, 14902–14907. doi:10.1073/pnas.0907740106

Riley, E.M., Schneider, G., Sambou, I., Greenwood, B.M., 1989. Suppression of cell-mediated immune responses to malaria antigens in pregnant Gambian women. Am. J. Trop. Med. Hyg. 40, 141–4.

Rodhain, J., 1940. Les plasmodium des anthropoids de l'Afriqe centrale et leurs relations avec les plasmodiums humains. Ann. Soc. Belg. Med. Trop. 35, 69–73.

Rodhain, J., 1939. Les plasmodiums des anthropoïdes de l'Afrique centrale et leurs relations avec les plasmodiums humains. Ann. Soc. Belge Méd. Trop. 19.

Rodhain, J., 1936. Contribution a l'étude des plasmodiums des singes africains. Ann. Soc. Belg. Med. Trop. 237–253.

Rodhain, J., Dellaert, R., 1955. Contribution a l'etude du Plasmodium schwetzi E. Brumpt (2me note). Transmission du Plasmodium schwetzi a l'homme. Ann. Soc. Belg. Med. Trop. 35, 73–76.

Rodhain, J., Dellaert, R., 1943. L'infection a Plasmodium malariae du chimpanze chez l'homme. Etude d'une premiere souche isolee de l'anthropoide Pan satyrus verus. Ann. Soc. Belg. Med. Trop. 23, 19–46.

Rogerson, S.J., Hviid, L., Duffy, P.E., Leke, R.F.G., Taylor, D.W., 2007. Malaria in pregnancy: pathogenesis and immunity. Lancet Infect. Dis. 7, 105–117. doi:10.1016/s1473-3099(07)70022-1

Roy, S., Vandenberghe, L.H., Kryazhimskiy, S., Grant, R., Calcedo, R., Yuan, X., Keough, M., Sandhu, A., Wang, Q., Medina-Jaszek, C.A., Plotkin, J.B., Wilson, J.M., 2009. Isolation and characterization of adenoviruses persistently shed from the gastrointestinal tract of non-human primates. PLoS Pathog. 5, e1000503. doi:10.1371/journal.ppat.1000503

Rudicell, R.S., Holland Jones, J., Wroblewski, E.E., Learn, G.H., Li, Y., Robertson, J.D., Greengrass, E., Grossmann, F., Kamenya, S., Pintea, L., Mjungu, D.C., Lonsdorf, E. V, Mosser, A., Lehman, C., Collins, D.A., Keele, B.F., Goodall, J., Hahn, B.H., Pusey, A.E., Wilson, M.L., 2010. Impact of simian immunodeficiency virus infection on chimpanzee population dynamics. PLoS Pathog. 6, e1001116. doi:10.1371/journal.ppat.1001116

Rudicell, R.S., Piel, A.K., Stewart, F., Moore, D.L., Learn, G.H., Li, Y., Takehisa, J., Pintea, L., Shaw, G.M., Moore, J., Sharp, P.M., Hahn, B.H., 2011. High prevalence of simian immunodeficiency virus infection in a community of savanna chimpanzees. J. Virol. 85, 9918–28. doi:10.1128/JVI.05475-11

Rynkiewicz, E.C., Pedersen, A.B., Fenton, A., 2015. An ecosystem approach to understanding and managing within-host parasite community dynamics. Trends Parasitol 31, 212–221. doi:10.1016/j.pt.2015.02.005

Sabbatani, S., Manfredi, R., Fiorino, S., 2010. Malaria infection and human evolution. Infez Med 18, 56–74.

Sadeuh-Mba, S.A., Bessaud, M., Joffret, M.L., Endegue Zanga, M.C., Balanant, J., Mpoudi Ngole, E., Njouom, R., Reynes, J.M., Delpeyroux, F., Rousset, D., 2014. Characterization of Enteroviruses from non-human primates in cameroon revealed virus types widespread in humans along with candidate new types and species. PLoS Negl Trop Dis 8, e3052. doi:10.1371/journal.pntd.0003052

Salanti, A., Dahlbäck, M., Turner, L., Nielsen, M.A., Barfod, L., Magistrado, P., Jensen, A.T.R., Lavstsen, T., Ofori, M.F., Marsh, K., Hviid, L., Theander, T.G., 2004. Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. J. Exp. Med. 200, 1197–203. doi:10.1084/jem.20041579

Santiago, M.L., Bibollet-Ruche, F., Bailes, E., Kamenya, S., Muller, M.N., Lukasik, M., Pusey, A.E., Collins, D.A., Wrangham, R.W., Goodall, J., Shaw, G.M., Sharp, P.M., Hahn, B.H., 2003a. Amplification of a complete simian immunodeficiency virus genome from fecal RNA of a wild chimpanzee. J. Virol. 77, 2233–2242. doi:10.1128/JVI.77.3.2233-2242.2003

Santiago, M.L., Lukasik, M., Kamenya, S., Li, Y., Bibollet-Ruche, F., Bailes, E., Muller, M.N., Emery, M., Goldenberg, D.A., Lwanga, J.S., Ayouba, A., Nerrienet, E., McClure, H.M., Heeney, J.L., Watts, D.P., Pusey, A.E., Collins, D.A., Wrangham, R.W., Goodall, J., Brookfield, J.F.Y., Sharp, P.M., Shaw, G.M., Hahn, B.H., 2003b. Foci of endemic simian immunodeficiency virus infection in wild-living eastern chimpanzees (Pan troglodytes schweinfurthii). J. Virol. 77, 7545–62.

Schaer, J., Perkins, S.L., Decher, J., Leendertz, F.H., Fahr, J., Weber, N., Matuschewski, K., 2013. High diversity of West African bat malaria parasites and a tight link with rodent Plasmodium taxa. Proc. Natl. Acad. Sci. U. S. A. 110, 17415–9. doi:10.1073/pnas.1311016110

Schielzeth, H., Forstmeier, W., 2009. Conclusions beyond support: overconfident estimates in mixed models. Behav Ecol 20, 416–420. doi:10.1093/beheco/arn145

Schubert, G., Stockhausen, M., Hoffmann, C., Merkel, K., Vigilant, L., Leendertz, F.H., Calvignac-Spencer, S., 2014. Targeted detection of mammalian species using carrion fly-derived DNA. Mol Ecol Resour. doi:10.1111/1755-0998.12306

Schwetz, J., 1934. Contribution à l'étude des parasites malariens des singes supérieurs africains. Rev. di Malariol. 13.

Scuda, N., Madinda, N.F., Akoua-Koffi, C., Adjogoua, E.V., Wevers, D., Hofmann, J., Cameron, K.N., Leendertz, S.A.J., Couacy-Hymann, E., Robbins, M., Boesch, C., Jarvis, M.A., Moens, U., Mugisha, L., Calvignac-Spencer, S., Leendertz, F.H., Ehlers, B., 2013. Novel polyomaviruses of nonhuman primates: genetic and serological predictors for the existence of multiple unknown polyomaviruses within the human population. PLoS Pathog. 9, e1003429. doi:10.1371/journal.ppat.1003429

Seimon, T.A., Olson, S.H., Lee, K.J., Rosen, G., Ondzie, A., Cameron, K., Reed, P., Anthony, S.J., Joly, D.O., McAloose, D., Lipkin, W.I., 2015. Adenovirus and herpesvirus diversity in free-ranging great apes in the Sangha region of the Republic Of Congo. PLoS One 10, e0118543. doi:10.1371/journal.pone.0118543

Sharp, C.P., LeBreton, M., Kantola, K., Nana, A., Diffo Jle, D., Djoko, C.F., Tamoufe, U., Kiyang, J.A., Babila, T.G., Ngole, E.M., Pybus, O.G., Delwart, E., Delaporte, E., Peeters, M., Soderlund-Venermo, M., Hedman, K., Wolfe, N.D., Simmonds, P., 2010. Widespread infection with homologues of human parvoviruses B19, PARV4, and human bocavirus of chimpanzees and gorillas in the wild. J Virol 84, 10289–10296. doi:10.1128/jvi.01304-10

Sharp, P.M., Hahn, B.H., 2010. The evolution of HIV-1 and the origin of AIDS. Philos Trans R Soc L. B Biol Sci 365, 2487–2494. doi:10.1098/rstb.2010.0031

Sharp, P.M., Rayner, J.C., Hahn, B.H., 2013. Evolution. Great apes and zoonoses. Science 340, 284–6. doi:10.1126/science.1236958

Shehzad, W., Riaz, T., Nawaz, M.A., Miquel, C., Poillot, C., Shah, S.A., Pompanon, F., Coissac, E., Taberlet, P., 2012. Carnivore diet analysis based on next-generation sequencing: application to the leopard cat (Prionailurus bengalensis) in Pakistan. Mol Ecol 21, 1951–1965. doi:10.1111/j.1365-294X.2011.05424.x

Sinka, M.E., Bangs, M.J., Manguin, S., Coetzee, M., Mbogo, C.M., Hemingway, J., Patil, A.P., Temperley, W.H., Gething, P.W., Kabaria, C.W., Okara, R.M., Van Boeckel, T., Godfray, H.C., Harbach, R.E., Hay, S.I., 2010. The dominant Anopheles vectors of human malaria in Africa, Europe and the Middle East: occurrence data, distribution maps and bionomic precis. Parasit Vectors 3, 117. doi:10.1186/1756-3305-3-117

Skarbinski, J., James, E.M., Causer, L.M., Barber, A.M., Mali, S., Nguyen-Dinh, P., Roberts, J.M., Parise, M.E., Slutsker, L., Newman, R.D., 2006. Malaria surveillance--United States, 2004., MMWR. Surveillance summaries : Morbidity and mortality weekly report. Surveillance summaries / CDC.

Smith, R., Wickings, E.J., Bowman, M.E., Belleoud, A., Dubreuil, G., Davies, J.J., Madsen, G., 1999. Corticotropin-releasing hormone in chimpanzee and gorilla pregnancies. J Clin Endocrinol Metab 84, 2820–2825.

Snounou, G., Viriyakosol, S., Zhu, X.P., Jarra, W., Pinheiro, L., do Rosario, V.E., Thaithong, S., Brown, K.N., 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 61, 315–320.

Sundararaman, S.A., Liu, W., Keele, B.F., Learn, G.H., Bittinger, K., Mouacha, F., Ahuka-Mundeke, S., Manske, M., Sherrill-Mix, S., Li, Y., Malenke, J.A., Delaporte, E., Laurent, C., Mpoudi Ngole, E., Kwiatkowski, D.P., Shaw, G.M., Rayner, J.C., Peeters, M., Sharp, P.M., Bushman, F.D., Hahn, B.H., 2013. Plasmodium falciparum-like parasites infecting wild apes in southern Cameroon do not represent a recurrent source of human malaria. Proc. Natl. Acad. Sci. U. S. A. 110, 7020–5. doi:10.1073/pnas.1305201110

Symondson, W.O., 2002. Molecular identification of prey in predator diets. Mol Ecol 11, 627–641.

Taberlet, P., Waits, L.P., Luikart, G., 1999. Noninvasive genetic sampling: look before you leap. Trends Ecol Evol 14, 323–327.

Takem, E.N., D'Alessandro, U., 2013. Malaria in pregnancy. Mediterr. J. Hematol. Infect. Dis. 5, e2013010. doi:10.4084/MJHID.2013.010

Tan, T.M.C., Noviyanti, R., Syafruddin, Marzuki, S., Ting, R.C.., 2000. NCBI. Direct submission.

Tanabe, K., Mita, T., Jombart, T., Eriksson, A., Horibe, S., Palacpac, N., Ranford-Cartwright, L., Sawai, H., Sakihama, N., Ohmae, H., Nakamura, M., Ferreira, M.U., Escalante, A.A., Prugnolle, F., Bjorkman, A., Farnert, A., Kaneko, A., Horii, T., Manica, A., Kishino, H., Balloux, F., 2010. Plasmodium falciparum accompanied the human expansion out of Africa. Curr Biol 20, 1283–1289. doi:10.1016/j.cub.2010.05.053

Tangpukdee, N., Duangdee, C., Wilairatana, P., Krudsood, S., 2009. Malaria diagnosis: a brief review. Korean J Parasitol 47, 93–102. doi:10.3347/kjp.2009.47.2.93

Tao, Y., Shi, M., Conrardy, C., Kuzmin, I. V, Recuenco, S., Agwanda, B., Alvarez, D.A., Ellison, J.A., Gilbert, A.T., Moran, D., Niezgoda, M., Lindblade, K.A., Holmes, E.C., Breiman, R.F., Rupprecht, C.E., Tong, S., 2013. Discovery of diverse polyomaviruses in bats and the evolutionary history of the Polyomaviridae. J. Gen. Virol. 94, 738–748. doi:10.1099/vir.0.047928-0

Tarello, W., 2005. A fatal Plasmodium reichenowi infection in a chimpanzee ? Rev. Méd. Vét. 156, 503–505.

Taylor, D.W., Wells, R.A., Vernes, A., Rosenberg, Y.J., Vogel, S., Diggs, C.L., 1985. Parasitologic and immunologic studies of experimental Plasmodium falciparum infection in nonsplenectomized chimpanzees (Pan troglodytes). Am J Trop Med Hyg 34, 36–44.

Taylor, P.G., 1996. Reproducibility of ancient DNA sequences from extinct Pleistocene fauna. Mol Biol Evol 13, 283–285.

Taylor, S.M., van Eijk, A.M., Hand, C.C., Mwandagalirwa, K., Messina, J.P., Tshefu, A.K., Atua, B., Emch, M., Muwonga, J., Meshnick, S.R., Ter Kuile, F.O., 2011. Quantification of the burden and consequences of pregnancy-associated malaria in the Democratic Republic of the Congo. J Infect Dis 204, 1762–1771. doi:10.1093/infdis/jir625

Tazi, L., Ayala, F.J., 2011. Unresolved direction of host transfer of Plasmodium vivax v. P. simium and P. malariae v. P. brasilianum. Infect Genet Evol 11, 209–221. doi:10.1016/j.meegid.2010.08.007

Telfer, S., Lambin, X., Birtles, R., Beldomenico, P., Burthe, S., Paterson, S., Begon, M., 2010. Species interactions in a parasite community drive infection risk in a wildlife population. Science (80-.). 330, 243–246. doi:10.1126/science.1190333

Therneau, T., 2012. coxme: Mixed Effects Cox Models. R Packag. version 2.2-3.

Thurber, M.I., Ghai, R.R., Hyeroba, D., Weny, G., Tumukunde, A., Chapman, C.A., Wiseman, R.W., Dinis, J., Steeil, J., Greiner, E.C., Friedrich, T.C., O'Connor, D.H., Goldberg, T.L., 2013. Co-infection and cross-species transmission of divergent Hepatocystis lineages in a wild African primate community. Int. J. Parasitol. 43, 613–9. doi:10.1016/j.ijpara.2013.03.002

Trimnell, A.R., Kraemer, S.M., Mukherjee, S., Phippard, D.J., Janes, J.H., Flamoe, E., Su, X.Z., Awadalla, P., Smith, J.D., 2006. Global genetic diversity and evolution of var genes associated with placental and severe childhood malaria. Mol Biochem Parasitol 148, 169–180. doi:10.1016/j.molbiopara.2006.03.012

UNESCO, 2015. Taï National Park - UNESCO World Heritage Centre [WWW Document]. URL http://whc.unesco.org/en/list/195 (accessed 6.26.15).

Vaidya, A.B., Akella, R., Suplick, K., 1989. Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. Mol Biochem Parasitol 35, 97–107.

Van Duivenvoorde, L.M., Voorberg-van der Wel, A., van der Werff, N.M., Braskamp, G., Remarque, E.J., Kondova, I., Kocken, C.H., Thomas, A.W., 2010. Suppression of Plasmodium cynomolgi in rhesus macaques by coinfection with Babesia microti. Infect Immun 78, 1032–1039. doi:10.1128/IAI.00921-09

Van Heuverswyn, F., Li, Y., Bailes, E., Neel, C., Lafay, B., Keele, B.F., Shaw, K.S., Takehisa, J., Kraus, M.H., Loul, S., Butel, C., Liegeois, F., Yangda, B., Sharp, P.M., Mpoudi-Ngole, E., Delaporte, E., Hahn, B.H., Peeters, M., 2007. Genetic diversity and phylogeographic clustering of SIVcpzPtt in wild chimpanzees in Cameroon. Virology 368, 155–71. doi:10.1016/j.virol.2007.06.018

Verhulst, N.O., Smallegange, R.C., Takken, W., 2012. Mosquitoes as potential bridge vectors of malaria parasites from non-human primates to humans. Front Physiol 3, 197. doi:10.3389/fphys.2012.00197

Verrelli, B.C., Tishkoff, S.A., Stone, A.C., Touchman, J.W., 2006. Contrasting histories of G6PD molecular evolution and malarial resistance in humans and chimpanzees. Mol Biol Evol 23, 1592–1601. doi:10.1093/molbev/msl024

Vestheim, H., Jarman, S.N., 2008. Blocking primers to enhance PCR amplification of rare sequences in mixed samples - a case study on prey DNA in Antarctic krill stomachs. Front Zool 5, 12. doi:10.1186/1742-9994-5-12

VILLESEN, P., 2007. FaBox: an online toolbox for fasta sequences. Mol. Ecol. Notes 7, 965–968. doi:10.1111/j.1471-8286.2007.01821.x

Volkman, S.K., Barry, A.E., Lyons, E.J., Nielsen, K.M., Thomas, S.M., Choi, M., Thakore, S.S., Day, K.P., Wirth, D.F., Hartl, D.L., 2001. Recent origin of Plasmodium falciparum from a single progenitor. Science (80-.). 293, 482–484. doi:10.1126/science.1059878

Voorberg-vd Wel, A., Kocken, C.H., Zeeman, A.M., Thomas, A.W., 2008. Detection of new Babesia microti-like parasites in a rhesus monkey (Macaca mulatta) with a suppressed Plasmodium cynomolgi infection. Am J Trop Med Hyg 78, 643–645.

Walker-Abbey, A., Djokam, R.R.T., Eno, A., Leke, R.F.G., Titanji, V.P.K., Fogako, J., Sama, G., Thuita, L.H., Beardslee, E., Snounou, G., Zhou, A., Taylor, D.W., 2005. Malaria in pregnant Cameroonian women: the effect of age and gravidity on submicroscopic and mixed-species infections and multiple parasite genotypes. Am. J. Trop. Med. Hyg. 72, 229–35.

Wallis, J., 1997. A survey of reproductive parameters in the free-ranging chimpanzees of Gombe National Park. J Reprod Fertil 109, 297–307.

WCF, 2012. ANNUAL REPORT 2012 Activities of the Wild Chimpanzee Foundation for improved conservation of chimpanzees and their habitat in West Africa.

Wevers, D., Metzger, S., Babweteera, F., Bieberbach, M., Boesch, C., Cameron, K., Couacy-Hymann, E., Cranfield, M., Gray, M., Harris, L.A., Head, J., Jeffery, K., Knauf, S., Lankester, F., Leendertz, S.A., Lonsdorf, E., Mugisha, L., Nitsche, A., Reed, P., Robbins, M., Travis, D.A., Zommers, Z., Leendertz, F.H., Ehlers, B., 2011. Novel adenoviruses in wild primates: a high level of genetic diversity and evidence of zoonotic transmissions. J Virol 85, 10774–10784. doi:10.1128/jvi.00810-11

White, N.J., 2003. Malaria, 21st ed, Manson's Tropical Diseases . W.B. Saunders.

Whitworth, J., Morgan, D., Quigley, M., Smith, A., Mayanja, B., Eotu, H., Omoding, N., Okongo, M., Malamba, S., Ojwiya, A., 2000. Effect of HIV-1 and increasing immunosuppression on malaria parasitaemia and clinical episodes in adults in rural Uganda: a cohort study. Lancet 356, 1051–1056. doi:10.1016/s0140-6736(00)02727-6

WHO, 2015. Malaria - Fact sheet #94 [WWW Document]. doi:http://www.who.int/mediacentre/factsheets/fs094/en/index.html

Williams, T.N., 2006. Red blood cell defects and malaria. Mol Biochem Parasitol 149, 121–127. doi:10.1016/j.molbiopara.2006.05.007