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NEW APPROACHES

A Cautionary Note on Fecal Sampling and Molecular Epidemiology in Predatory Wild Great Apes

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Fecal samples are an important source of information on parasites (viruses, prokaryotes, or eukaryotes) infecting wild great apes. Molecular analysis of fecal 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, for example, another non-human primate. To determine the potential magnitude of this issue, we estimated the prevalence of prey DNA in fecal samples obtained from two wild chimpanzee communities. We observed values >15%, which are higher than or close to the fecal detection rates of many great ape parasites. Contamination of fecal 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. Am. J. Primatol. 77:833–840, 2015.

Key words: non-invasive; chimpanzee feces; infectious disease; carnivore; parasites; microorganism

INTRODUCTION

An increasing number of studies rely on non-invasively collected samples to investigate parasites infecting great apes and other primates (here and in the following parasite designates any infectious microorganism, whether viral, prokaryotic, or eukaryotic). This is often done by searching for nucleic acid fragments of parasites in fecal samples. The detection and/or sequencing of such fragments can allow for the discovery of novel parasites, 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 I).

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]. Parasite nucleic acids found in feces could as well originate from parasites infecting prey and thus reflect the diet (ingestion and excretion) rather than infection status of the host. For instance, in a recent study on malaria parasite infection in wild chimpanzees, we found that 20% of the fecal samples from which malaria parasite sequences were obtained actually contained *Hepatocystis* sp. sequences [De Nys et al., 2014]. *Hepatocystis* sp. are malaria parasites which 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], 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 feces, which we could test for [De Nys et al., 2014]. However, when knowledge about parasites 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

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TABLE I. Examples of Prevalence of Microorganisms Estimated Non-Invasively Between 2003 and 2014

Microorganism	Prevalence (%)	References
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., 2014]
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	[Santiago et al., 2003]
Simian immunodeficiency virus[3]	30.0	[Santiago et al., 2003]
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]

Most prevalence estimates are based on nucleic acid detection and/or antibody detection in fecal samples from wild chimpanzees and represent the proportion of positive samples or infected individuals. [1]Prevalence estimates for a combination of several non-human primate species, [2]combined results from tissue samples and fecal samples,[3]Combined results from fecal and urine samples.

chimpanzee feces could be of plant or animal origin. Moreover, parasite prevalence as estimated through fecal detection rates (i.e. proportions of fecal samples which test positive) tends to be relatively low in chimpanzees, mostly falling <20% among 30 studies performed between 2003 and 2014 (Fig. 1 and Table I). Many studies are thus likely to be significantly impacted by even low-frequency contamination with prey parasite genetic material. Also, chimpanzees sometimes prey on other non-human primate (NHP) species [Boesch and Boesch-Achermann, 2000]. Due to the close evolutionary relationships between primate prey and chimpanzees, primate prey may host parasites closely related to those infecting chimpanzees, and lead to a misinterpretation of the results.

How unaccounted-for "contamination" of feces with the DNA of parasites infecting the prey will influence the interpretation of fecal epidemiology in predatory great apes depends on hunting frequency and parasite 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, parasite 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 fecal 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

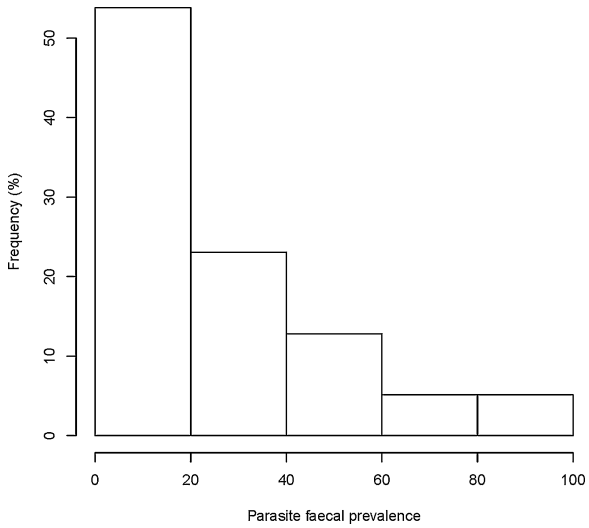

Fig. 1. Frequency plot of parasite fecal prevalence determined in 30 studies from 2003 to 2014. All studies focused on parasites infecting wild chimpanzees. A complete list of these studies and the according prevalence can be found in Table I.

methods to detect prey DNA in fecal samplings of two distinct chimpanzee communities, using fecal 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 fecal epidemiology studies. We propose that molecular detection of prey prevalence be used as a control for diet-induced parasite detection in carnivorous great apes. This will simultaneously provide information on hunting frequency (directly and even where apes are not habituated) and possible prey parasite genetic material persistence (indirectly, through prey genetic material persistence).

METHODS

Study Populations and Sample Collection

One set of fecal samples ($n = 107$) was collected from one habituated group of wild western chimpanzees (*Pan troglodytes verus*, Taï National Park, 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 fecal samples ($n = 109$) was collected from non-habituated wild central chimpanzees (*Pan troglodytes 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, for example, NHP, duikers, tortoises (NFM, MR, and CB personal communication).

Finally, a third set of fecal samples ($n = 92$) collected in Loango from western lowland gorillas (*Gorilla gorilla 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 feces. A first molecular study of gorilla diet, also in Loango, identified mammal DNA sequences in their feces [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.

This research complied with protocols approved by the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique and the Office Ivoirien des Parcs et Réserves in Côte d'Ivoire; and the Agence Nationale des Parcs Nationaux and Centre National de la Recherche Scientifique et Technologique in Gabon. It adhered to the legal requirements of the countries in which the work took place and to the American Society of Primatologists Principles for the Ethical Treatment of Non-Human Primates.

Molecular Analysis

DNA was extracted from fecal 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 II; [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], and added a blocking primer [Shehzad et al., 2012; Vestheim and Jarman, 2008] to prevent the amplification of great

TABLE II. Primers Used in This Study

Target	Targeted sequence	Approx. fragment length (bp)	Primer name	Sequence (5′-3′)	Reference
Mammal	mt 16S DNA	130	16Smam1_f	CGGTTGGGGTGAC CTCGGA	[Taylor , 1996]
			16Smam3_r	CTCGATGTTGGATC AGGACATC	[Calvignac Spencer et al. , 2013]
			16Smam_blkhum3	CGGTTGGGGCGACC TCGGAGCAGAACCC—spacerC3	[Boessenkool et al. , 2012]
			16Smam_blkpig	CGGTTGGGGTGACC TCGGAGTACAAAAAAC—spacerC3	[Calvignac Spencer et al. , 2013]
Colobinae	mt 12S rRNA	122	Colo_f	CGATTGACCCGAGCTAATAGRY	[Schubert et al. , 2014]
			Colo_r	CCACTTTCGTAGTTTA TTTTACATTG	[Schubert et al. , 2014]
Colobinae	mt CR	480	L15449Clbs	CCRCCAATA CCCAAAACTGG	[Minhos et al. , 2013]
			H15973Clb	AGGAGAGTAGCA CTCTTGTGC	[Minhos et al. , 2013]

mt: mitochondrial.

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 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), 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 our 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 sec at 95°C, 30 sec at 64°C, 60 sec 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, 1994; 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).

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 fecal samples, respectively, as well as in 2.2% of the Loango gorilla fecal 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; Fig. 2). Most were identified as red colobus ($n = 20$), including the six samples which had tested positive

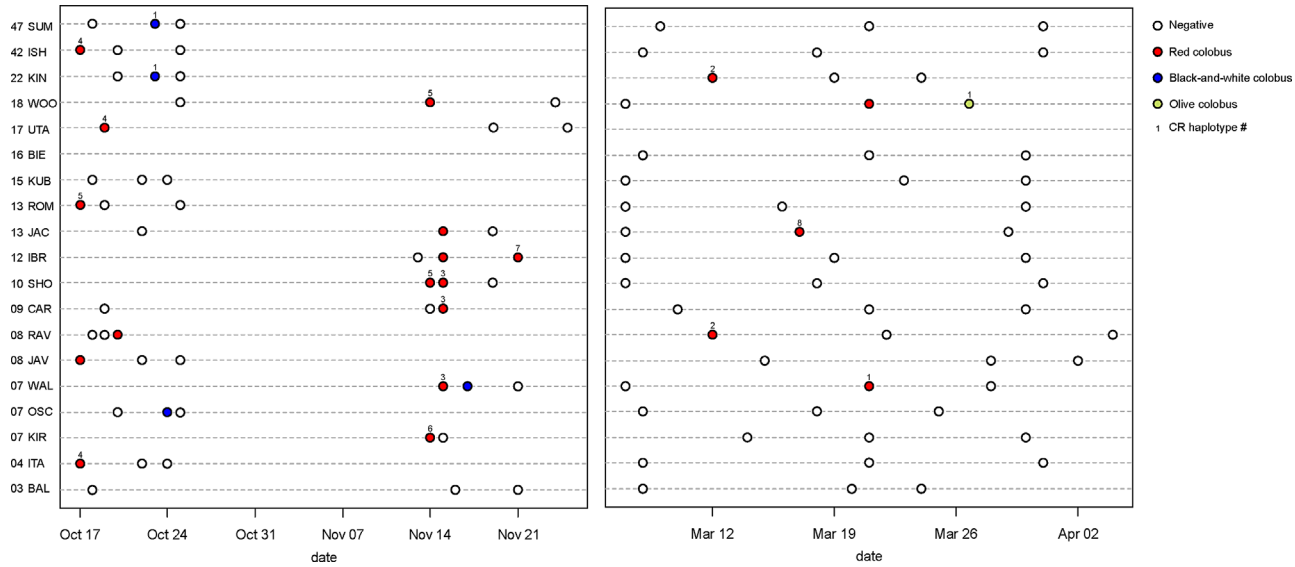

Fig. 2. Prey detection through time in Taï. Individual chimpanzees are represented by a three letter abbreviation, preceded by their age at time of sampling. Mitochondrial control region (CR) haplotypes are identified by numbers above the corresponding samples. Note that the CR fragment could not be amplified from all positive samples (a likely side-effect of it being much larger than for the other colobine-specific system that we used).

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 (Fig. 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 (Fig. 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: 7–41%). Based on this sensitivity, we extrapolated that prey prevalence in chimpanzee feces from Loango was 15.3% (95% confidence interval: 9–52.4%).

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 fecal detection rates of parasites in studies performed between 2003 and 2014 (Fig. 1 and Table I). Therefore, our findings certainly have implications for molecular epidemiology based on fecal sampling.

There are two main factors that will determine whether a study is at risk of being biased by unaccounted-for detection of prey parasites. As just discussed, the prevalence of the parasite of interest in feces is of importance. If it is high, and sensitive molecular detection methods are used, parasite 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 parasite fecal prevalence is of importance. If parasite fecal prevalence is low, parasite 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 parasite and prey detection are independent. This could be achieved by determining prey detection rates in parasite-positive and a subset of parasite-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 parasite of interest is likely to infect chimpanzees depends, however, on a second factor: how much is known about the parasite 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 parasite detection rates can be trusted to mostly reflect chimpanzee infection. A good example here is simian immunodeficiency viruses infecting chimpanzees (SIVcpz). Fecal 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 fecal prevalence. Here, controlling for diet-induced parasite detection is unnecessary. On the contrary, in cases where parasites are known to frequently undergo host switches and/or to simultaneously exploit several host species or when nothing is known about the association of this parasite and their host(s), detection of prey parasite should be ruled out to reinforce the belief in an association between the newly detected parasite 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 fecal 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, that is, 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 fecal 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 toward 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 toward a low rate of laboratory work-induced false positives: (i) Taï chimpanzees are known to prey on the mammal species detected [Boesch, 1994; 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, 1994; 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 toward 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 parasite molecular epidemiology based on fecal 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 parasite. Parasite 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 feces 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.

CONCLUSION

In this article, we demonstrate that predatory ape fecal samples often test positive for prey DNA. Depending on parasite fecal prevalence and the extent of prior knowledge about host-parasite associations, this should prompt microbiologists, parasitologists and virologists who use fecal 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 behavioral research on predatory apes [Gogarten et al., 2014], as the data generated will both help in identifying meaningful predatory ape-parasite associations and in describing their diets.

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