IJP: Parasites and Wildlife 10 (2019) 59–70

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Differences in infection patterns of vector-borne blood-stage parasites of sympatric Malagasy primate species (*Microcebus murinus*, *M. ravelobensis*)

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ARTICLE INFO

Keywords:
Microcebus spp.
Onchocercidae
Microfilaria
Evolution
Ecology
Madagascar

ABSTRACT

The dynamic relationship of vector-borne parasites, arthropod vectors and their hosts is prone to change under the influence of climate change, global integration, shifting demographics and deforestation. It is therefore essential to better understand parasitism in wildlife populations, including parasites transmitted by blood-feeding vectors, and explore host range and heterogeneity of parasitic infections. We investigated Giemsa stained blood smears of two sympatric Malagasy primate species (*Microcebus murinus*: 184 samples from 69 individuals and *M. ravelobensis*: 264 samples from 91 individuals) for blood-stage parasites and tested for a potential influence of host species, sex, body mass and sampling month on blood-stage parasite prevalence and infection intensity. No protozoan parasites were detected in either host species. A host-specific difference was observed in filarial nematode infections, with higher risk of infection in *M. murinus* (prevalence 30.43%), than in *M. ravelobensis* (prevalence 6.59%), which may be explained by differences in host behavior and/or immune competence, linked to the period of host-parasite coevolution. Neither sex nor sampling month influenced infection prevalence or intensity significantly. We did not observe a negative effect of microfilarial infections on host fitness when taking body mass as a proxy. Our results support the hypothesis of a long-term evolutionary adaptation of hosts and parasites, leading to persistent infection with low morbidity. Morphological and molecular analyses indicate the finding of a new species, "*Lemurfilaria lemuris*". Genetic analysis furthermore showed > 99% sequence identity with microfilariae described from a sympatric, larger-bodied lemur species of a different genus, suggesting low host-specificity of the detected filariae and pathogen transmission across genus boundaries. Findings contribute to a more comprehensive picture of vector-borne diseases of Malagasy lemurs.

1. Introduction

Blood-stage parasites, such as *Plasmodium*, *Babesia* and *Theileria* species and filarial nematodes, are the causative agents of devastating diseases like malaria, babesiosis, filariasis and river blindness in humans, east coast fever in livestock or canine heartworm disease in companion animals. Moreover, they comprise several zoonotic parasites, e.g. *Babesia divergens* (Gray et al., 2010), *Plasmodium knowlesi* (Jongwutiwes et al., 2004) and *Dirofilaria repens* (McCall et al., 2008). All of these parasitic diseases are transmitted by arthropod vectors, which are especially abundant in tropical climates. Wildlife populations can play an important role as potential reservoirs for these infectious agents. More than 70% of emerging infectious diseases in recent decades have originated from animals (Jones et al., 2008). Non-human primates are of specific importance regarding pathogen spillover due to their relatedness to humans (Wolfe et al., 2007). Continuous human encroachment into wildlife habitats may increase infection pressure and potential spread of vector-borne pathogens from humans or livestock to wildlife and vice versa (Patz et al., 2000). Therefore, it is important to increase our knowledge on parasite species richness and prevalence in wildlife, to understand patterns of parasitic infections, possible pathways of pathogen transmission and identify potential threats to endangered wildlife species. It is furthermore essential to investigate heterogeneity of parasitic infections in multi-host communities and identify potential factors that may lead to differences in susceptibility or morbidity in different host species (Fenton and Pedersen, 2005; Fenton et al., 2015).

Mouse lemurs (genus: *Microcebus*) are the smallest primates in the world and are endemic to the island of Madagascar. They are a nocturnal, forest-dwelling, cryptic genus, with currently 24 described

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https://doi.org/10.1016/j.ijppaw.2019.07.003
Received 2 April 2019; Received in revised form 5 July 2019; Accepted 6 July 2019

species (Radespiel et al., 2012; Rasoloarison et al., 2013; Zimmermann and Radespiel, 2014; Hotaling et al., 2016). The isolated evolution of wildlife on Madagascar over the past 90 million years has generated a unique fauna and flora, making the island one of the world's biodiversity hotspots today. However, poverty, political instability and human population growth have led to destruction and conversion of lemur habitats for anthropogenic use and the majority of Madagascar's primate species are now classified as Endangered by the IUCN red list (Schwitzer et al., 2014; Jones et al., 2019). Unlike the intensely studied mammalian groups, a great part of the invertebrate fauna of Madagascar remains yet to be described (Goodman and Benstead, 2005). The possible cascading effects of species loss also render several parasite species endangered and this coextinction further enhances biodiversity loss (Koh et al., 2004).

Infections with *Babesia* spp., *Plasmodium* spp., trypanosomes and filarial nematodes have been described in several, mostly larger-bodied, Malagasy lemur hosts (Uilenberg et al., 1972; Irwin and Raharison, 2009; Springer et al., 2015; Larsen et al., 2016; Hokan et al., 2017). This is the first systematic investigation of blood-stage parasites in two sympatric small-bodied lemur species, the Golden-brown Mouse Lemur (*Microcebus ravelobensis*) and the Grey Mouse Lemur (*M. murinus*), in consideration of potential factors that may contribute to heterogeneity of infection.

The two study species have a comparable small body mass of about 60g with no sexual dimorphism, and live in partial sympatry in the strongly seasonal dry deciduous forests of northwestern Madagascar (Zimmermann et al., 1998). The area is considered a center of endemism where temporary isolation during glacial periods led to regional speciation events (Wilmé et al., 2006). Genetic data indicates an evolutionary origin of *M. ravelobensis* in this region, whereas *M. murinus* shows signs of a more recent spatial expansion into this region (Yoder et al., 2000; Schneider et al., 2010). *Microcebus murinus* and *M. ravelobensis* are promiscuous seasonal breeders (Schmelting, 2000), with the onset of reproductive activity preceding the rainy season, and show a great dietary flexibility (Thorén, 2011). Both mouse lemur species are known to forage solitarily at night but show distinct socioecological differences regarding daytime sleeping sites. *Microcebus ravelobensis* sleeps in mixed-sex sleeping groups in relatively open vegetation. In contrast, M. *murinus* prefers the confined space of tree holes, and the degree of sociality differs between the sexes (Radespiel et al., 2003). Male *M. murinus* usually sleep solitarily, while females are often found in stable sleeping groups of related individuals (Radespiel et al. 1998, 2001). This unique setting of two model species, comparable in size, diet and activity patterns, that are exposed to the same environmental conditions, allows to explore a potential influence of sociality and sleeping site ecology on blood-stage parasite infections.

The effect of sociality on the risk of infection with vector-borne pathogens is controversial. Group-living may have protective properties through an encounter-dilution effect but could also attract vectors and thereby increase the probability of infection (Patterson and Ruckstuhl, 2013). Primates, and particularly mouse lemurs, spend more than half of their lives in sleeping sites (McNamara et al., 2010). The protected wooden shelter of a tree hole does not only provide thermoregulatory benefits (Lutermann et al., 2010), but may also give some degree of protection from flying, diurnal insects, and other exophagous pathogen vectors and thereby reduce the probability of infection with insect-borne parasites, such as filarial nematodes or malaria parasites (Nunn and Heymann, 2005). At the same time, the microhabitat of a tree hole represents a favorable breeding ground for mosquito species (Mitchell and Rockett, 2017) and an advantageous environment for temporary parasites such as ticks. A previous investigation on mouse lemur ectoparasites in northwestern Madagascar (Ankarafantsika National Park) found a higher risk of tick infestation in tree-hole dwelling *M. murinus* than in *M. ravelobensis* (Klein et al., 2018).

On the evolutionary scale, the relationship of host and parasite is shaped by an arms race between host immune defense and parasite evasion strategies (Behnke et al., 1992; Schmid-Hempel, 2011). It has therefore been argued that invasive parasites show higher virulence in endemic species that lack the resistance the original host has gained via co-evolution (naive host syndrome (Mastitsky et al., 2010)). Consequently, differences in susceptibility to parasitic infections in sympatric host species may derive from differences in host-parasite co-evolution (Wells and Clark, 2019).

The aim of this study was to enhance the current state of knowledge on parasitism in Malagasy primates and explore potential drivers of heterogeneous infection patterns. We investigated blood-stage parasites of the two sympatric small-bodied mouse lemur species, *M. murinus* and *M. ravelobensis,* over the course of two study periods in northwestern Madagascar to characterize parasite species richness, prevalence and infection intensity. Comparison of infection patterns within and between the two host species and in different seasonal settings enables us to explore potential factors influencing blood-stage parasite infections, such as climatic conditions, host sex and host species. Climatic conditions are known to influence vector activity and parasite development (Altizer et al., 2006), and we therefore expected an increase in infection intensity and, to a lesser extent, blood-stage parasite prevalence in the hot and humid period (March, April and November) in both hosts. Immunosuppressive properties of the hormone testosterone have been discussed as predisposing for parasitic infections (Klein, 2004; Roberts et al., 2004). We therefore predicted a higher prevalence and infection intensity in males than in females. Based on the differences in sleeping behavior described above, we expected a higher prevalence of tick-borne parasites in *M. murinus*, while an effect of sleeping site ecology on parasites transmitted via flying vectors may be less distinct. Further, a potential shorter period of host-parasite co-evolution may render *M. murinus* more susceptible to blood- transmitted parasites, leading to higher prevalence and infection intensity.

2. Material and methods

2.1. Sample collection

The study was conducted in the forest area Jardin Botanique A (JBA, 16°19′S, 46°48′E) of the Ankarafantsika National Park in northwestern Madagascar. The dry deciduous forest in this area is subjected to seasonally changing climatic conditions with a hot and humid rainy season from November to April and a relatively cooler dry season from May to October (Klein et al., 2018). A total of 160 mouse lemurs (91 *M. ravelobensis* and 69 *M. murinus*) were captured using Sherman live traps from April to November 2015 and from March to May 2016. Captured animals were weighed, sexed and individually marked upon first capture with a small subcutaneous transponder (Trovan ID-100; Telinject®, Römerberg, Germany) that allows lifelong identification. Blood samples were taken once per month via puncture from the saphenous vein. The emerging blood drop was collected with a pipette and 3 µl were used to create a thin smear on a glass slide on site that was fixed with methanol. Additional blood (if present) was collected, stored in RNAlater solution and kept frozen at −20 °C until further processing. The majority of animals (58%) contributed more than one data point to the final data set of 448 blood smears (184 *M. murinus*, 264 *M. ravelobensis*) and 291 RNAlater samples (106 *M. murinus*, 185 *M. ravelobensis*). In April 2016, the dead body of one of the study animals, a female *M. murinus*, was detected in the forest and collected for a postmortem examination.

2.2. Laboratory analyses

2.2.1. Examination of blood smears

Blood smears were stained with Giemsa solution upon return to Germany and screened with an Axiophote microscope (Carl Zeiss MicroImaging, Jena, Germany) for microfilaria. Parasites were photographed with a Colorview IIIu Camera and measured using the command “polyline” in cell^B Image Acquisition Software (version 3.1;

Olympus Soft Imaging Solutions, Hamburg, Germany). In addition, 100 000 red blood cells of one blood smear per individual (in total 160 blood smears) were inspected under 100-fold magnification using oil emersion to screen for intraerythrocytic protozoan parasites.

2.2.2. Necropsy of an adult M. murinus

One microfilaremic female *M. murinus*, that had been sampled regularly since June 2015, was found dead in April 2016. The necropsy revealed a fractured skull, suggesting trauma to the head as the most likely cause of death. An adult helminth specimen was discovered in the abdominal cavity, dorsal of the bladder, which was collected and stored in 90% ethanol. After measurement of the specimen, the anterior and posterior extremities were embedded in polyvinyl-lactophenol for morphologic examination, while the intermediate segment was used for DNA extraction for further genetic analysis. Parasite morphology was compared to available descriptions of filarial nematodes occurring in Madagascar (Chandler, 1929; Chabaud and Choquet, 1955; Chabaud et al. 1961, 1965).

2.2.3. Molecular genetic analysis of helminth parasites

DNA from the helminth specimen (see 2.2.2) was isolated using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and eluted in 100 µl double-distilled water. In addition, DNA was extracted from a blood sample of a male *M. murinus* collected in August 2015 and stored in 0.5 ml RNAlater solution, using the NucleoSpin® Blood Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and again eluted in 100 µl double-distilled water.

The ITS1–5.8S–ITS2 rDNA region of microfilariae in the blood sample was amplified using primers NC5 and NC2 (Gasser et al., 1996) in a 50 µl reaction mixture containing 30 µl double-distilled water, 1 µl dNTPs (10 mM each), 2 µl of each primer (10 µM each), 5 µl 10 x buffer, 2 µl Taq polymerase (Dream Taq, Thermo Fisher Scientific, Epsom, United Kingdom) and 10 µl DNA of the microfilaremic blood sample as template. For the adult helminth specimen, the reaction mixture was adjusted to 2 µl DNA template by increasing the double-distilled water to 38 µl. Thermocycling conditions were as follows: 95 °C for 3 min for initial denaturation followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s, and final extension at 72 °C for 10 min.

A 704 bp fragment of the cytochrome oxidase subunit I gene (COI) of the adult helminth specimen was furthermore amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994) in a 25 µl reaction mixture containing 18.5 µl double-distilled water, 0.5 µl dNTPs (10 mM each), 1 µl for/rev primer (10 µM each), 2.5 µl 10x buffer, 0.5 µl Taq Polymerase (5 Prime, Hilden, Germany) and 1 µl DNA template. Thermocycling was performed using a touch-down profile including an initial denaturation at 94 °C for 3 min, followed by 3 cycles at 95 °C for 30 s, 55 °C (−1 °C per cycle) for 30 s and 72 °C for 60 s, followed by 37 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 60 s, and final extension at 72 °C for 10 min.

A fragment of the 18S rRNA of the adult helminth was amplified in a 25 µl reaction mixture corresponding to the recipe used for the COI using primers Ns1 and Ns2a (Barker et al., 2003). The thermocycling protocol corresponded to Klein et al. (2018).

The amplified fragments were inserted into pCR®4-TOPO® vectors and transformed into One Shot®TOP10 chemically competent *E. coli* (TOPO® TA cloning kit for sequencing; Invitrogen, Karlsruhe, Germany). Plasmid DNA was obtained using the NucleoSpin® Plasmid Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's recommendations and Sanger-sequenced at Microsynth Seqlab Sequence Laboratories (Göttingen, Germany). The PCR product of the 18S rRNA fragment was directly sent for sequencing. Obtained nucleotide sequences were compared to publicly available sequences via BLAST search and aligned using Geneious v10.2.6 (Biomatters Ltd., Auckland, New Zealand). A phylogenetic tree was constructed using the Maximum Likelihood method (Tamura-Nei model) based on a 351 bp fragment of the ITS1 sequence including the top 20 sequences obtained from NCBI GenBank (acc. no. XR002251420 was excluded due to low query cover) in MEGA7 software (Tamura et al., 2007) and bootstrap analyses were performed with 1000 replicates. An additional phylogenetic tree was constructed based on COI sequences for broader taxonomic classification following Lefoulon et al., 2015. Sequences were deposited in NCBI GenBank under accession numbers MK060112, MK060113, MN119552 and MN129783.

2.3. Statistical analysis

Total prevalence was calculated for filarial nematodes in both mouse lemur species by identifying an individual as positive if at least one smear contained microfilaria over the course of the whole study period. Difference in prevalence between species was tested by using Chi-Square test. Individual monthly presence/absence data for microfilaria in each blood smear was then analyzed in more detail by fitting a generalized linear model (GLM) with logit-link and binomial assumption. The following fixed effects (predictive variables) were tested: host species (*M. murinus*, *M. ravelobensis*), sex, body mass (pregnant females excluded), sampling time and the temporal factor month (March – November). A comparison of April-May 2015 and April-May 2016, the two periods sampled in both years, did not reveal differences between the study years and data was therefore pooled for April and May. For eight individuals that were captured in the same month of both years, the sample from 2015 was excluded from the analysis, as information on sampling time and body mass was missing. Sampling times were categorized as: early morning: 08h00 – 10h00, late morning: 10h00 – 12h00, noon: 12h00 – 14h00, early afternoon: 14h00 – 16h00 and late afternoon: after 16h00. The final model was compared to a null model in a likelihood ratio test and model fit was compared based on the Akaike information criterion (AIC). Body mass was then modelled as a response variable to the fixed effects microfilaria, microfilaria/µl, month and species, including the interaction term microfilaria * month, since body mass in mouse lemurs is known to vary over the course of the year (Klein et al., 2018).

Furthermore, potential influences of the same predictive variables ("species", "sex", "body mass" "month" and "sampling time") on the number of microfilariae/µl (microfilaremia) were tested by including all positive samples in a multiple linear regression model (LM). Fixed factors were set as in the GLM described above and microfilaria counts were log-transformed to achieve a normal distribution. GLM and LM models for the predictive variables "sampling time" and "body mass" were analyzed separately, as they were not fitted to the same dataset, since 19 (4.32%) and 57 (12.95%) samples had to be excluded for the analysis of body mass and sampling time, respectively, due to missing values.

The length of measured microfilariae was analyzed in a linear mixed effects model (LMM) with the predictive variables host species, sex and month and inclusion of a grouping factor "animal ID" as a random-effect term to account for repeated measurements within one sample. The predictive variable month was subjected to post-hoc analysis, computing all pairwise differences between months in a Tukey's test. Statistics were performed in R version 3.5.2 (R Core Team, 2018) using the packages nlme (Pinheiro et al., 2014), lme4 (Bates et al., 2014) and multcomp (Hothorn et al., 2008).

3. Results

3.1. Morphological findings

Morphologic examination of the male adult helminth specimen revealed a total length of 36.48 mm and width of 478 µm. The anterior end was rounded with a peribuccal, rectangular structure and the nerve ring was located 382 µm from the anterior end. Caudal papillae were not clearly recognizable. The tail measured 443.5 µm. Spiculae length

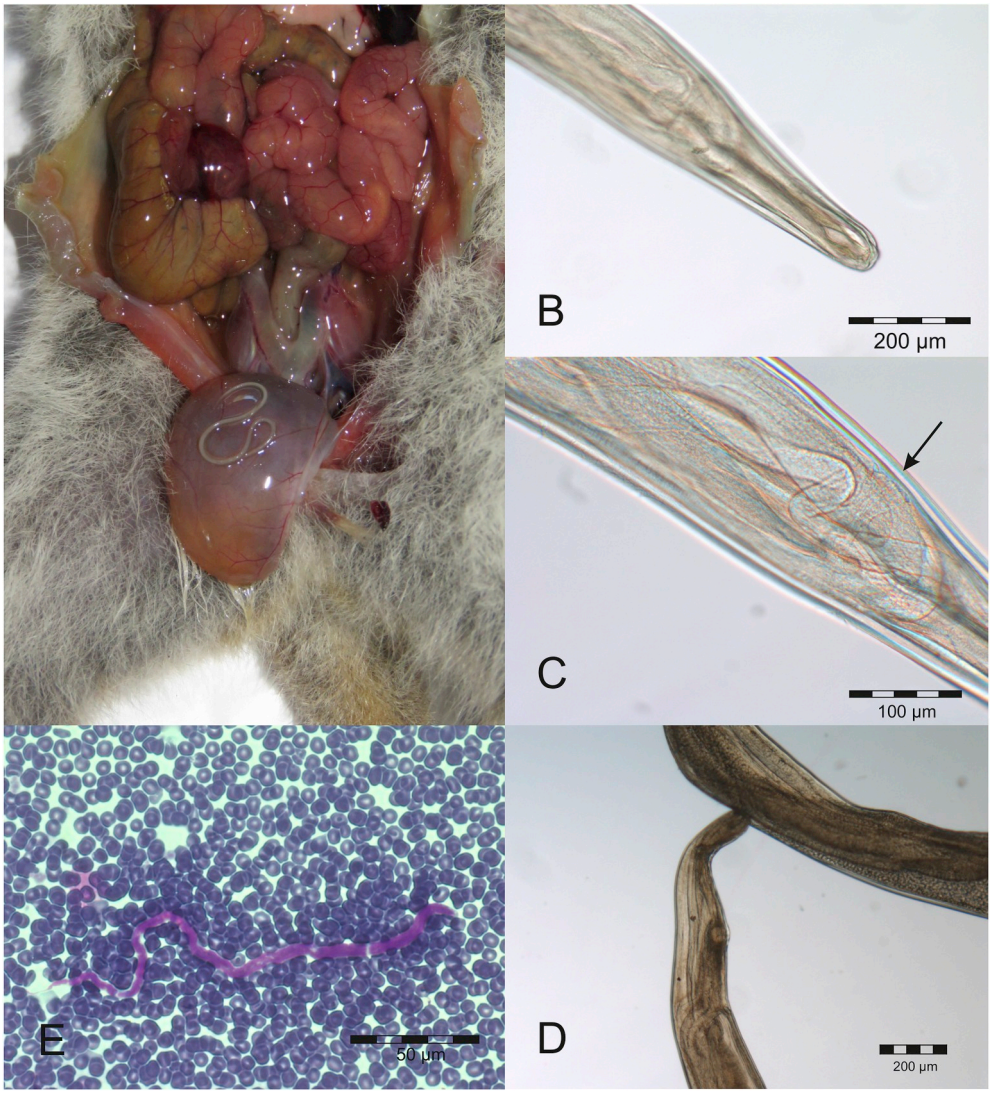

Fig. 1. Filarial nematodes from a *M. murinus* host: (A) Adult male filarial nematode specimen in situ, (B) anterior end of the adult specimen, (C) anterior region with nerve ring, (D) intermediate section and posterior end of the adult specimen and (E) microfilaria in a blood smear.

could not be clearly distinguished (Fig. 1, Table 1). Based on these results, the specimen could not be assigned to any described Malagasy filarial nematode species.

A total of 499 microfilariae were measured (466 from *M. murinus*, 33 from *M. ravelobensis*) and length ranged from 157.25 to 274.77 µm, with a mean length of 225.91 ± 19.53 µm. Microfilaria length did not differ significantly between the two host species (*M. murinus*: 226.42 ± 19.53 µm, *M. ravelobensis*: 218 ± 18.33 µm) or different sexes (female: 220.42 ± 18.71 µm, male: 229.93 ± 19.16 µm), but monthly variations in microfilaria length were statistically significant. Overall, microfilariae were on average shorter in March and longer in July, September and October (Fig. 2, Supplementary file 1).

3.2. Blood-stage parasite prevalence and intensity

No protozoan parasites (such as *Plasmodium* spp., *Trypanosoma* spp. or *Babesia* spp.) were detected in the screened Giemsa-stained blood smears. However, filarial nematodes were detected in blood smears of both mouse lemur species. Trapping frequency and capture success varied greatly between different months, leading to unequal sample distribution with overrepresentation of the dry months (Fig. 3). The total prevalence of microfilariae was 30.43% in *M. murinus* (n = 69) and only 6.59% in *M. ravelobensis* (n = 91). This species difference proved statistically significant in a Chi-Square test ($\chi^2 = 14.25$, $P < 0.001$), and was significant in the GLM (Table 2). Infection intensity ranged from 0.33 to 21.33 microfilariae/µl and was significantly higher in *M. murinus* (0.33–21.33, mean 3.98) than in *M. ravelobensis* (0.33–3.67, mean 1.22) (Table 2). The courses of infection of 17 individuals that were sampled multiple times are displayed in Fig. 4. Of the 27 animals that were tested positive for filarial nematodes in this study, six no longer had microfilariae in their blood smears at a later time point. However, two of these six animals were again tested positive afterwards, whereas the other four remained negative. The necropsied *M. murinus* was first microfilaremic in September 2015 and microfilariae were consistently detected in her blood smears subsequently, with the last sample taken two days prior to the date when she was found dead. There was no significant influence of host sex, sampling month or time of blood collection on microfilaria prevalence or intensity (Table 2). The risk of infection was significantly higher in individuals with higher body mass, but body mass did not influence

Table 1
Morphometric comparison of the filarial nematode species found in this study with other Onchocercidae spp. previously described in Malagasy lemurs.

Species	Host species	Adult ♂	Microfilaria
Dipetalenoma petteri (Chabaud and Choquet, 1955)	*Lepilemur ruficaudatus* (in captivity)	not described	Length: 285 µm
Paulianfilaria puliani (Chabaud et al., 1961)	*Propithecus verreauxi*	Length: 23–29 mm, Width: 300 µm, Nerve ring: 285 µm from the anterior end, Tail: 165 µm, without lateral alae, 7 pericloacal papillae, 2 pairs of papillae at the posterior third of the tail, 1 pair of papillae at the terminus, Spiculae length: left 375 µm, right 125 µm	Length: 230 µm
Courduriella courdurieri (Chabaud et al., 1961)	*Indri indri*	Length: 21 mm, Width: 125 µm, Nerve ring: 135 µm from the anterior end, Tail: 70 µm, small ventro-lateral caudal alae, Pericloacal papillae small: 1 anterior to the cloaca, 5 pairs around lateral and posterior borders of the cloaca, 1 pair of papillae at the posterior third of the tail, Spiculae length: left 360 µm, right 95 µm	Length: 128–140 µm
Protofilaria furcata (Chandler, 1929)	*Varecia rubra* (in captivity), *Hapalemur griseus*	Length: 13–15 mm, Width: 150–175 µm, Nerve ring: 270 µm from the anterior end, Tail: 95–100 µm, Pericloacal papillae small and difficult to discern, Spiculae length: left 98 µm, right 78 µm	Length: 250–277 µm
Lemurfilaria lemuris (this study)/ Onchocercidae sp. (Hokan et al., 2017)	*Microcebus murinus*, *Lepilemur edwardsi*	Length: 36.48 mm, Width: 478 µm, Nerve ring: 382 µm from the anterior end, Tail: 443.5 µm, Caudal papillae not clearly recognizable, Spicluae length: not clearly distinguishable	Length: 177–226 µm

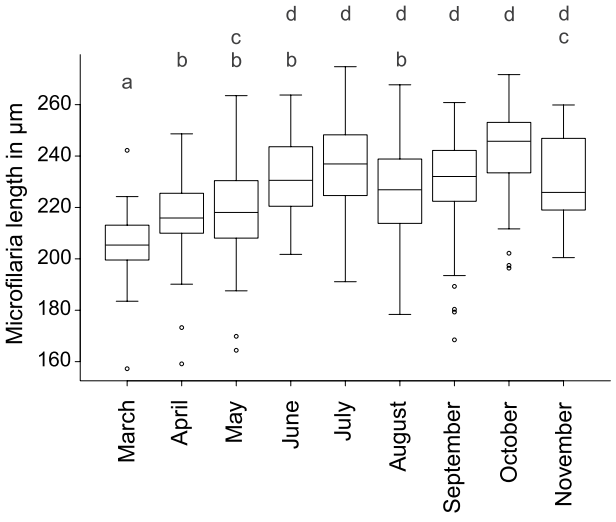

Fig. 2. Microfilaria length across the different sampling months. Different small letters above the box plots indicate statistical differences ($P < 0.05$) in microfilaria length between sampling months.

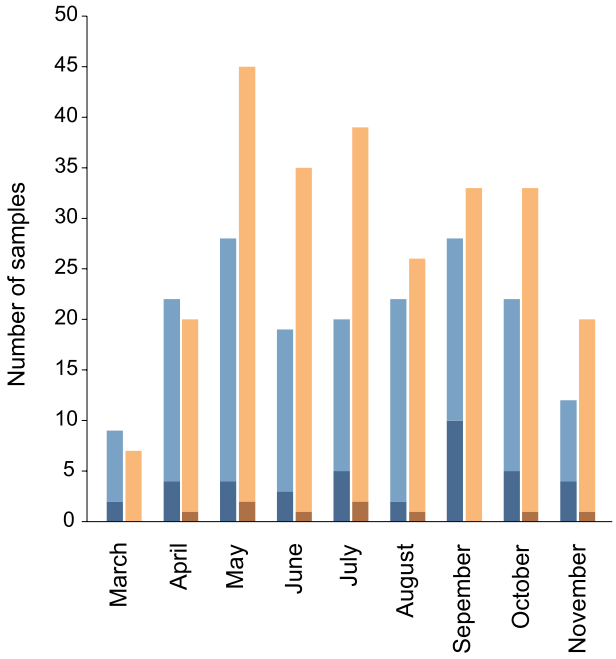

Fig. 3. Number of samples (blood smears) per month. Microfilaria positive samples are shown in dark blue for *M. murinus* and dark brown for *M. ravelobensis*, microfilaria negative samples in light blue for *M. murinus* and light brown for *M. ravelobensis*. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

microfilaremia intensity (Table 2). Body mass in turn was influenced by sampling month and species, but neither by presence of microfilaria nor number of microfilaria/µl (Table 3).

3.3. Genetic comparison of the adult helminth specimen and microfilaria

Comparison of the 1361 bp fragment of the ITS1–5.8S–ITS2 rDNA region sequenced from the microfilaremic blood sample of a male *M. murinus* taken in August 2015 (GenBank acc. no. MK060112) with a 1360 bp fragment amplified from the adult helminth collected from the abdominal cavity of a female *M. murinus* in April 2016 (acc. no. MK060113) showed 99.41% identity. Blast results furthermore revealed 99.49 and 99.56% identity (query cover 100%) with an unnamed Onchocercidae sp. sequenced from the blood sample of a *Lepilemur edwardsi* (acc. no. KY586137, Hokan et al., 2017). In a phylogenetic analysis, the two sequences generated in this study unambiguously nested within a clade of Onchocercidae spp. infecting lemurs (Fig. 5). Neither comparison of the 18S rRNA (acc. no. MN129783) nor the COI fragment (acc. no. MN119552) with publicly

Table 2
Results of generalized linear models (GLM, logit link, binomial assumption), and multiple linear regression models (LM) for potential factors influencing microfilaria prevalence and intensity.

Model	factor	Estimate	Std. Error	*Df*	*P* value	Effect on microfilaria
Prevalenc						
	(Likelihood ratio test comparing the full model to a null model: Deviance = 42.405, Df = 10, *P* = < 0.001)					
1 (N = 440)						
	Species	−2.077	0.39	1	< 0.001***	*M. ravelobensis* < *M. murinus*
	Sex	−0.342	0.329	1	0.3	
	month			8		
	August	−0.608	0.785		0.439	
	July	0.382	0.656		0.56	
	June	−0.257	0.732		0.726	
	March	−0.093	0.929		0.921	
	May	−0.19	0.664		0.774	
	November	0.661	0.722		0.36	
	October	0.133	0.672		0.843	
	September	0.562	0.617		0.363	
2 (N = 383)	Time of sampling[a]	−0.156	0.206	4	0.449	No influence of sampling time point
3 (N = 421)	Body mass[b]	0.201	0.084	1	0.017*	Significant influence of body mass
Intensity						
4 (N = 48)	(Likelihood ratio test comparing the full model to a null model: χ^2 = 19.093, Df = 10, *P* = 0.063)					
	Species	−1.032	0.402	1	0.014 *	*M. ravelobensis* < *M. murinus*
	Sex	0.541	0.314	1	0.094	Male = female
	Month			8		
	August	−0.29	0.735		0.695	
	July	−0.156	0.58		0.789	
	June	−0.274	0.657		0.68	
	March	0.121	0.825		0.885	
	May	−0.288	0.598		0.633	
	November	−1.152	0.622		0.072	
	October	−0.975	0.609		0.118	
	September	−0.902	0.546		0.107	
5 (N = 42)	Time of sampling[c]	−0.124	0.193	4	0.524	No influence of sampling time point
6 (N = 46)	Body mass[d]	0.072	0.102	1	0.486	No influence of body mass

Estimate: difference between categories at the logit scale.
Std.Error: standard error of the corresponding estimate based on the GLM/LM fit.
Significance codes: *** < 0.001, ** < 0.01, * < 0.05.
[a] Likelihood ratio test comparing the full model to a null model: Deviance = 46.561, Df = 11, *P* < 0.001.
[b] Likelihood ratio test comparing the full model to a null model: Deviance = 88.497, Df = 19, *P* < 0.001.
[c] Likelihood ratio test comparing the full model to a null model: χ^2 = 16.06, Df = 11, *P* = 0.23.
[d] Likelihood ratio test comparing the full model to a null model: χ^2 = 21.44, Df = 19, *P* = 0.391.

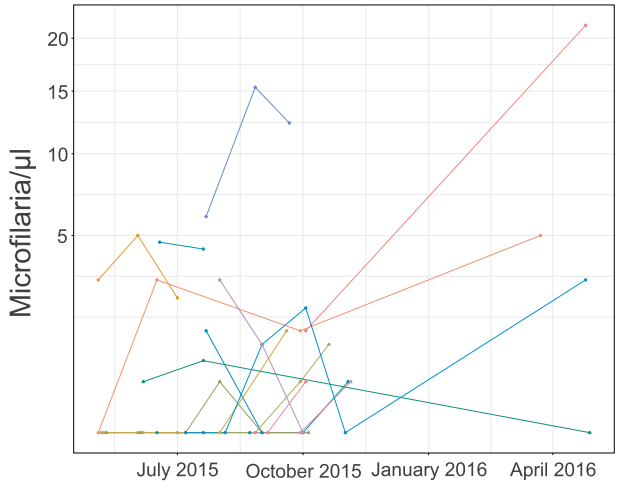

Fig. 4. Microfilaremia of individual mouse lemurs over time (points: *M. murinus*, triangles: *M. ravelobensis*).

Table 3
Results of the multiple linear regression model (LM) for potential factors influencing host body mass. Likelihood ratio test comparing the full model to a null model: χ^2 = 4507.2, F = 5.193, Df = 19, *P* < 0.001.

Factor	Estimate	Std. Error	*Df*	*P* value	Effect on microfilaria
Microfilaria	1.318	3.649	1	0.718	No influence of microfilarial infections
Microfilaria/µl	0.309	0.257	1	0.23	No influence of infection intensity
Species	2.637	0.72	1	0.001**	*M. ravelobensis* > *M. murinus*
Month			8		
August	−4.907	1.608		0.002**	
July	−6.604	1.569		< 0.001***	
June	−2.497	1.599		0.119	
March	0.405	2.201		0.854	
May	0.615	1.514		0.685	
November	−6.507	1.871		< 0.001***	
October	−5.04	1.584		0.002**	
September	−3.44	1.573		0.029*	

Estimate: difference between categories at the logit scale.
Std.Error: standard error of the corresponding estimate based on the LM fit.
Significance codes: *** < 0.001, ** < 0.01, * < 0.05.

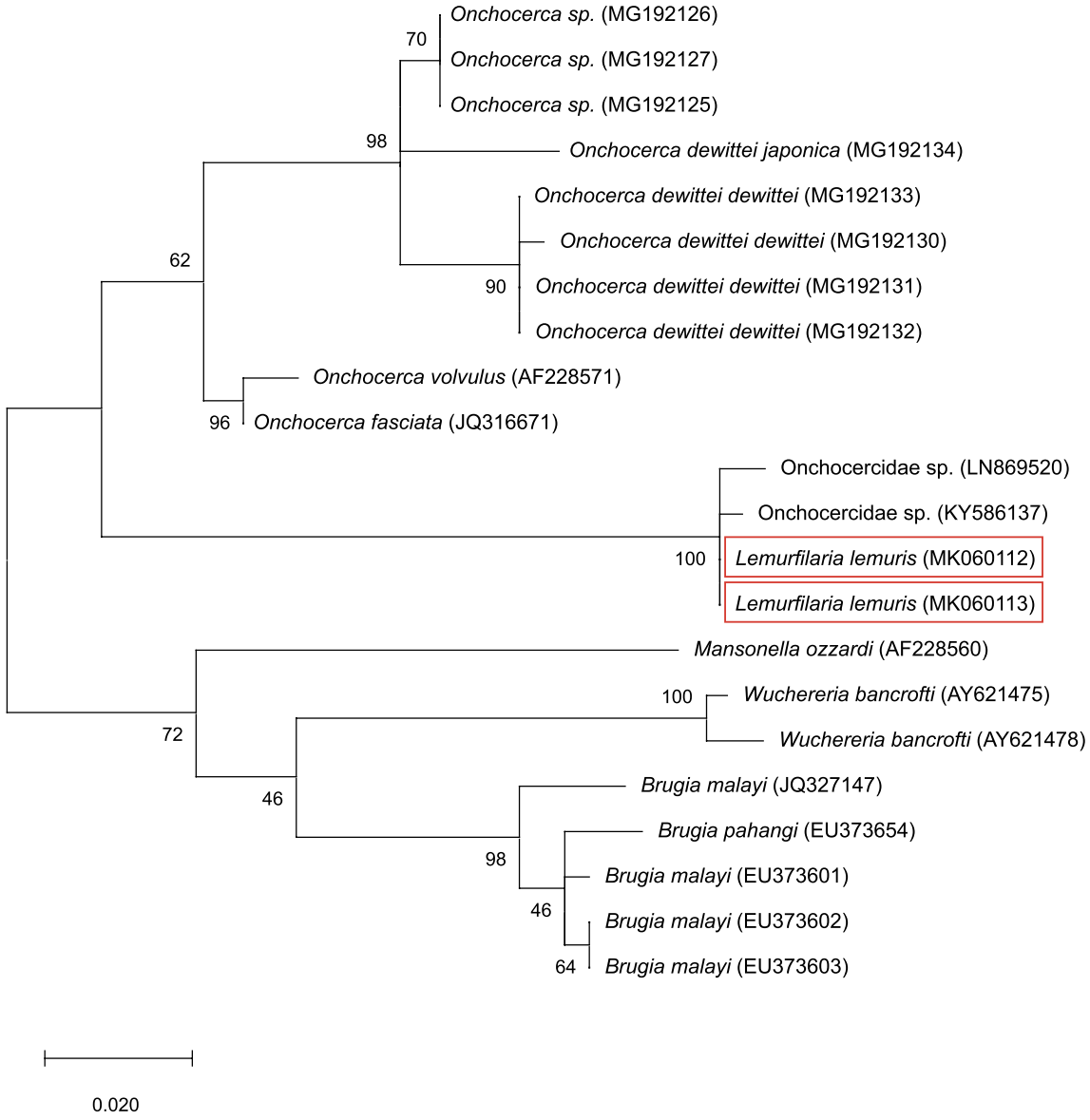

Fig. 5. Phylogenetic tree of Onchocercidae species constructed on the basis of partial ITS1 sequences using the Maximum Likelihood method. The percentage of replicate trees in which the associated species clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branch lengths is measured in the number of substitutions per site. The sequences of the present study are framed in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

available sequences enabled a conclusive genus assignment, but confirmed classification in the family: Onchocercidae (Fig. 6).

4. Discussion

This is the first empirical study on blood-stage parasites of two sympatric host species of the smallest primate radiation, the mouse lemurs. We did not detect protozoan parasites but *M. murinus* and *M. ravelobensis* were both identified as definitive hosts of a filarial nematode species with microfilariae circulating in the blood.

4.1. Methodological considerations

Four individuals, which had been identified as microfilaremic, no longer had microfilaria in their blood smears at subsequent captures. Two individuals furthermore tested negative for filarial nematodes in between positive sampling occasions. This unexpected change in infection status could be explained by the fact that low parasitaemia of less than 0.33 microfilaria/μl in the circulating blood may have remained unnoticed. An immunocompetent host may furthermore be able to eliminate circulating microfilariae (Maizels and Lawrence, 1991; McCall et al., 2008). A study on *Propithecus verreauxi* (Springer et al., 2015) combined microscopic examination of thin blood smears with PCR-based molecular detection methods for filarial nematodes in blood samples and neither method proved to be 100% sensitive. In 11% of cases, the PCR result was positive even though infection was not morphologically determined. Investigation of the peripheral blood furthermore only enables detection of circulating microfilaria. The preceding live cycle stages of the parasite from transmission of the infective larvae by a vector to maturation of the adult worm until reproduction and release of microfilaria pass unnoticed. We therefore also assume a certain proportion of false negative results in our study.

A cautious note is also warranted on the lack of evidence for protozoan parasites in this study. Examination of a combination of thick and thin Giemsa stained blood smears is considered the 'gold standard' for laboratory diagnosis of malaria in humans worldwide, mainly due to

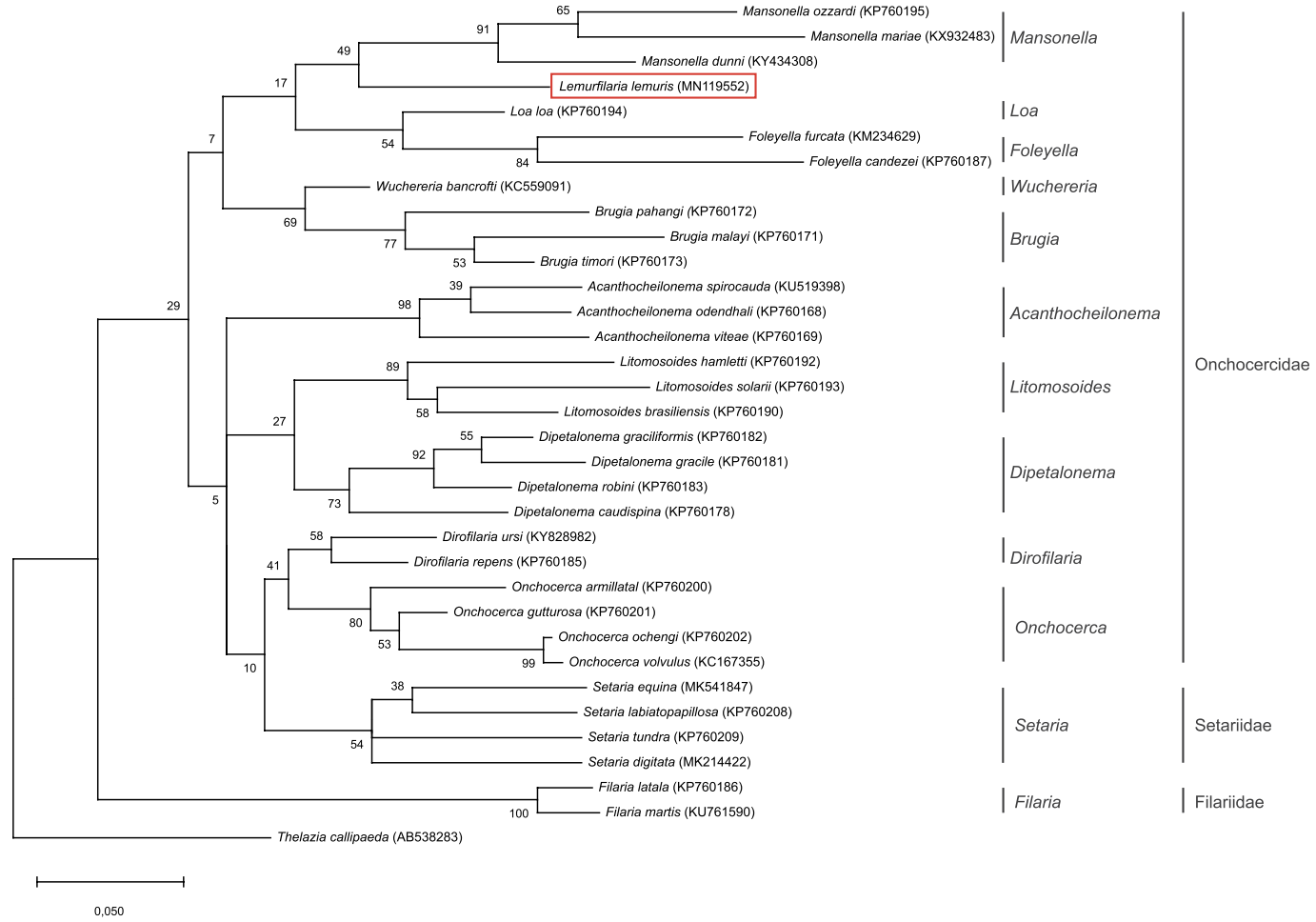

Fig. 6. Phylogenetic tree of 33 filarial nematode species constructed on the basis of partial COI sequences using the Maximum Likelihood method. The percentage of replicate trees in which the associated species clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Branch lengths is measured in the number of substitutions per site. *Thelazia callipaeda* was included as an outgroup. The sequence of the present study is framed in red.

the simplicity and low costs of this method. The thick smear provides the sensitivity of the test, enabling the experienced investigator to detect infections of 50–100 parasites/µl (Tangpukdee et al., 2009), while examination of thin films enables species identification. Since we only prepared thin smears, sensitivity was limited in our study and low levels of parasitemia may have gone unnoticed. In their study of hemoparasites in sifakas, Springer et al. (2015) could not detect any intraerythrocytic parasite stages in thin blood smears of individuals that were tested positive for *Babesia* and *Plasmodium* spp. by PCR. It is therefore possible that protozoan parasites were present in the studied *Microcebus* populations as well, but infection intensities may have been too low for detection via microscopy.

4.2. Filarial nematode identification

Four species of filarial nematodes, belonging to the family Onchocercidae, have been described in lemurs: *Dipetalonema petteri*, *Paulianfilaria pauliani*, *Courduriella courdurieri* and *Protofilaria furcata* (Chandler, 1929; Chabaud and Choquet, 1955; Chabaud et al. 1961, 1965; Irwin and Raharison, 2009). Their life cycle and mode of transmission are unstudied, but similar to other Onchocercidae adults they can be expected to reside in the thoracic and abdominal cavity of the definitive host and released microfilariae are ingested by blood sucking insect vectors as intermediate hosts. In the vector, microfilariae develop into L3 larvae, which are infective for the definitive mammalian host and are transmitted during subsequent blood meals (Anderson, 2000). Filarial nematodes have been described as parasites of several lemur species (Irwin and Raharison, 2009), and *M. murinus* has been listed as a host of *Dipetalonema petteri* (Chabaud et al., 1965). However, details on this record specifying sample collection, parasite species identification or development stage of the collected parasite could not be found.

In this study, we found more than 99% pairwise identity in the ITS1–5.8S–ITS2 sequence of an adult worm and microfilaria. An intraspecific p-distance for the ITS1 and ITS2 rDNA region from 0 to 2% and from 0 to 5.92% was identified in a different Onchocerca sp., *Onchocerca volvulus*, by Morales-Hojas et al. (2001). Accordingly, the sequence identity of the adult worm with that of the microfilaria from this study suggests that both belong to the same species. Identification of filarial nematodes to the species level based on morphological characteristics of microfilaria in thin blood smears is not very reliable, since different methods of blood smear fixation can lead to variations in microfilaria characteristics. Schacher (1962) found that microfilaria of *Brugia pahangi* shrank by 32.5% in air-dried alcohol-fixed blood smears when compared to formalin fixed wet mounts. Of the four filarial nematode species described in lemurs, the microfilariae measured in this study are in the range described for *P. pauliani*. However, the adult specimen found in this study did not match the morphological characteristics of *P. pauliani*, or any of the other Onchocercidae sp. that have been described in lemurs so far. The phylogeny of filarial nematodes is under constant revision in the light of new DNA barcoding approaches (Blaxter et al., 1998; Lefoulon et al., 2015). Several widely used genetic markers, such as COI or 18S rRNA fragments, allow distinction on the species level, but are not suitable to resolve phylogenetic relations (Lefoulon et al., 2015), complicating molecular genetic classification of

filarial nematodes. While comparison of the generated 18S rRNA and COI fragment of the adult specimen from this study with published sequences confirmed classification in the family of Onchocercidae, we did not get a conclusive species or even genus assignment. We therefore propose to designate the filarial nematode detected in this study as a putative new species, *"Lemurfilaria lemuris"*.

The amplified ITS1–5.8S–ITS2 DNA fragments furthermore showed 99.49–99.56% pairwise identity with a nucleotide sequence of microfilariae obtained from a larger bodied, hole- sleeping, sympatric lemur species of another genus, *L. edwardsi* (acc. no.: KY586137), that was sampled at the same study site (Hokan et al., 2017). This high sequence identity again indicates that both belong to the same filarial nematode species, suggesting low host specificity and pathogen transmission across genus boundaries. The degree of host specificity varies greatly between different filarial nematode species. *Onchocerca volvulus* for example is restricted to humans, whereas *B. malayi* has been reported in a wide range of hosts, including nonhuman primates, felids, canines and humans, and the zoonotic character of this parasite complicates the control of human infections (Dissanaike, 1979). Given the clear genetic differentiation of the filarial nematode found in this study from parasites known from humans or domestic animals a spillover event due to anthropogenic pressure, as recently described for the canine heartworm (*Dirofilaria immitis*), detected in *Microcebus rufus* in southeastern Madagascar (Zohdy et al., 2019), seems unlikely.

The significant difference in microfilaria length between samples from different months, as we found it in the present study, could indicate the presence of separate species or morphotypes at different times of the year, as it has been found in other lemur species (Springer et al., 2015; Hokan et al., 2017). However, we did not see a clear delineation between shorter and longer microfilariae which could be explained by inter- and intraspecific plasticity. We were able to generate a nucleotide sequence for longer microfilaria found in August. No DNA sample of shorter microfilariae from the late rainy season (March, April) could be amplified successfully. This may be due to low blood sample volumes and/or microfilaremia or a possible degradation of samples during storage or transport in the hot and humid rainy season.

4.3. Considerations regarding potential vectors of Lemurfilaria lemuris

All filarial nematodes require a competent vector as intermediate host in which microfilariae can develop into mammalian-infective L3 larvae to complete their life cycle. Known arthropod vectors are for example blood sucking flying insects of the order Diptera (e.g. Culicidae for *Dirofilaria* spp., *Wucheria* spp. and *Brugia* spp., or Simuliidae and Ceratopogonidae for *Onchocerca* spp.), wingless insects (e.g. fleas and lice for for *Acanthocheilonema* spp.) or Acari (e.g. mites for *Litomosoides* spp.) (Williams, 1948; Paily et al., 2009; Otranto et al., 2013). A previous investigation on ectoparasitic infestation of the mouse lemur populations sampled in this study revealed a significantly higher risk of infection with ticks for *M. murinus* compared to *M. ravelobensis*. The fact that *M. murinus* showed a higher prevalence for microfilaria in this study may therefore suggest that ticks could be potential vectors for *L. lemuris*. However, ticks are not common vectors for filarial nematodes and have so far only been identified as competent vectors for *Acanthocheilonema* (Olmeda-García et al., 1993) and potential vectors for *Cercopithifilaria* spp. (Otranto et al., 2012). Hokan et al., (2017) furthermore did not observe tick infestation in a sympatric larger lemur species, *Avahi occidentalis*, which showed a microfilaria prevalence of 66.7%. Comparing our results of the COI analyses to the comprehensive phylogenetic study of Lefoulon et al. (2015), *L. lemuris* would be affiliated with clade ONC5, a group of Onchocercidae comprised of several genera. While competent vectors for the human filariae included in this clade have been identified, the transmission routes for several animal associated species remain unknown. The current knowledge on vector range for different Onchocercidae genera would suggest a flying insect vector for the newly described *L. lemuris*, but empirical data will need to verify this hypothesis.

4.4. Circadian rhythms of filarial nematode infections

Several filarial nematodes show circadian rhythms in microfilaremia in the peripheral blood that is synchronized with vector activity (Paily et al., 2009). Therefore, we included time of sampling in our statistical analyses. However, based on our results we could not see variations in microfilaremia over the course of the day as it has also been shown for other filarial nematodes such as *Litomosoides carinii* and *Dipetalonema viteae* (Reddy et al., 1984). Captured animals were processed in the morning hours and most blood samples were therefore taken between 9h00 and 12h00, which does not coincide with the activity period of potential mosquito vectors since *Culex*, *Anopheles* and *Mansonia* spp., which are known to transmit microfilaria in Madagascar (Tantely et al., 2016), are active at night, dusk and dawn. Blood was furthermore taken from the saphenous vein, and not from the capillary periphery. A higher abundance of microfilaria in the periphery favors uptake by a bloodsucking vector and a circadian periodicity is therefore marked by microfilaria accumulation in cutaneous vessels at the time of vector activity (Barrozo et al., 2004). A more systematic sampling protocol at different time points including the night, dawn and dusk, and from the capillary periphery would allow for a better assessment of potential circadian rhythms.

4.5. Influence of host sex and body mass on microfilarial infection

A potential influence of host sex on parasitic infections is discussed controversially (Roberts et al., 2004). A sex bias has been documented in several filarial nematode species, with higher prevalence and infection intensities in males (Klein, 2004). These differences may be attributed to sex-specific differences in exposure to pathogens (Renz et al., 1987) or immunosuppressive properties of the hormone testosterone, rendering the host more susceptible to parasitic infection (Nakanishi et al., 1989; Hughes and Randolph, 2001; Merkel et al., 2007). However, no effect of sex on microfilaria prevalence or intensity was found in the present study. This result can be explained in two ways. First, varying exposure to mosquitoes, which are likely vectors for filarial nematodes, seems unlikely in the studied mouse lemur species, as they do not exhibit sexual dimorphism and both sexes forage solitarily at night. Second, mouse lemurs are seasonal breeders and males show high annual variation in plasma testosterone levels in synchrony with changes in testis size and reproductive activity (Petter-Rousseaux and Picon, 1981), and resistance to and clearance of parasitic infections may only be lower in the mating season due to elevated androgen levels. Filariae, however, are known to persist in their hosts for long periods of time. A temporary, sex-specific predisposition may therefore remain unnoticed.

Individual body mass was used as an indicator to investigate a potential relationship of microfilaria infections and host fitness, but we found a higher risk of infection in individuals with higher body mass. This may be explained by an underlying age effect. The prepatent period, meaning the time from infection with the infective stage larvae to maturation of the adult reproductive worm releasing microfilaria may span several months and young animals are therefore not expected to test positive for microfilaria even though they may already be prepatently infected. Indeed, for two individuals, captured for the first time as small-bodied subadults and then repeatedly across months, infection could not be detected before they reached sexual maturity (indicating a minimum age of 6 months) and a higher body mass. The observed influence of body mass on microfilaria prevalence may therefore well reflect the complex interplay of parasite life cycle and age composition of the host population with young individuals of lower body mass not showing microfilaria in the blood.

Body mass in turn was influenced by species and sampling month, with *M. ravelobensis* being overall heavier than *M. murinus*, and a marked decrease in body mass in both species over the course of the dry season, which is in accordance with a previous study on the same population (Klein et al., 2018). Body mass, however, did not respond to presence or absence of microfilaria. These findings could indicate low pathogenicity of the detected filaril nematode. The clinical characteristics of filarial infections vary significantly between different parasite species related to the tissue tropism of the respective pathogen. Adults of *Onchocerca flexuosa* or *D. repens* for example are found in the subcutaneous tissue of deer and dogs respectively, *Setaria digitata* in the abdominal cavity of deer (Mohanty et al., 2000; Hidalgo et al., 2015). They show low pathogenicity and infections can remain asymptomatic. Similar characteristics have been described for human filariae of the genus *Mansonella* (Ta-Tang et al., 2018). Other filarial nematode infections develop a chronic progression and accumulation of adult canine heartworms, *D. immitis*, can be fatal, but may remain unnoticed over extended periods until clinical manifestation (Simón et al., 2012). The low morbidity, assumed in our study, may be linked to a potential tissue tropism of *L. lemuris* to the abdominal cavity associated with low pathogenicity in the two studied host species. More detailed analyses, including possible fitness-related effects of this and other pathogens, such as ecto- and intestinal parasites, are needed to better understand the interplay of parasite load and fitness consequences.

4.6. Host specific differences in microfilarial infections

The risk of infection with microfilariae, as well as infection intensity, differed significantly between the two studied host species with higher prevalence and microfilaremia in *M. murinus* than in *M. ravelobensis*. Infections of vector-borne diseases are shaped, on the one hand, by host-vector contact, defining exposure to the respective pathogen, and on the other hand by the host's immunological response after parasite transmission. It has been argued that prevalence of filarial nematode infections may be influenced by the hosts' sleeping site ecology, since a better secluded sleeping site may provide some degree of protection from flying vectors and therefore counteract the risk of infection with filarial nematodes (Hokan et al., 2017). Known differences in sleeping site ecology of the two studied mouse lemur species, with *M. murinus* sleeping in the protected wooden shelter of tree holes and *M. ravelobensis* sleeping in more open vegetation, would therefore suggest a predisposition of *M. ravelobensis* for parasites transmitted by flying insect vectors and a corresponding higher microfilaria prevalence. The findings of this study, however, do not support this hypothesis. Contrary to the findings of Hokan et al. (2017), the lemur species using a less protective sleeping site had a significantly lower microfilaria prevalence in the present study. These contradictory results lead to the assumption that the risk of filarial nematode infections may not be linked to host sleeping site ecology. The frequent use of tree holes by different definitive host species, *M. murinus* and *L. edwardsi*, as previously observed at this study site (personal observation), may however increase vector- and thereby pathogen-exposure and contribute to filarial nematode transmission across species and genus boundaries.

Likely, mosquito vectors for microfilarial transmission, like *Culex*, *Anopheles* and *Mansonia* spp. (Tantely et al., 2016), are active at night, dusk and dawn, a time when both studied mouse lemur hosts forage solitarily in the forest. Leaving and seeking sleeping sites around dusk and dawn therefore coincides with peak mosquito activity and possibly increases vector-contact in both species. On the other hand, a study by Thorén et al. (2011) revealed seasonal changes in nocturnal activity patterns with a reduction of locomotor activity and increase of resting in female *M. murinus* during the night that was not observed in female *M. ravelobensis* during the early dry season. Such behavioral differences during the nocturnal activity period may potentially lead to dissimilar vector exposure, with resting individuals more prone to bites of flying insect vectors than active ones, and contribute to the difference in microfilaria prevalence of the two host species. Nevertheless, this remains speculative. Biting activity of potential alternative flying insect vectors, such as members of the family Simuliidae shows great variation (Barrozo et al., 2004) and has not been investigated in Madagascar. We hence lack the scientific basis for valid reasoning with regard to host-vector contact. Further parasitological studies are needed to identify the vector of *L. lemuris*. Studies with complementary detailed investigation of individual host activity patterns, sleeping group size and sociality would then be necessary to test for these behavioral factors or a potential encounter-dilution effect. *Microcebus murinus* is furthermore known to enter hibernation (i.e. prolonged periods of torpor), in response to low temperature and restricted water and food resources during the dry season in southeastern Madagascar (Schmid and Ganzhorn, 2009). Hibernation also results in adjustments in immunology, such as reduced white blood cell counts in the peripheral blood, which may render individuals more susceptible to infection (Martinez-Bakker and Helm, 2015). However, both, *M. murinus* and *M. ravelobensis* in the Ankarafantsika National Park, show seasonal daily torpor with no significant differences between the two species (Randrianambinina et al., 2003; Thorén et al., 2011). This factor therefore does not contribute to heterogeneity of filarial nematode infections.

Alternatively, the species difference in microfilaria prevalence and intensity may be explained by host-derived factors. Filarial infections are chronic diseases with parasites persisting and reproducing in the hostile environment of an immunocompetent host over extended periods of time. To investigate this parasite-host relationship in detail, a filariasis model of *Litomosoides sigmodontis* in mice has been established and studies found a strong influence of the genetic background of the host, with different mice strains differing in their susceptibility to injected *L. sigmodontis* (Petit et al., 1992). Hoffmann et al. (2001) furthermore found that rapid clearance of microfilaria was most likely mediated by the innate immune system, whereas elimination of microfilaria at later time points post infection was strongly influenced by MHC-related factors. Differences in genetic background and MHC-diversity could also be influencing susceptibility to filarial nematodes in the two studied mouse lemur species. Recent studies investigated the phylogeography of *Microcebus* species. The analysis of mitochondrial D-loop sequences showed signals of a relatively recent spatial expansion of *M. murinus* in northwestern Madagascar that should have coincided with a strong founder event (Schneider et al., 2010). *M. ravelobensis*, in contrast, evolved in the region comprising the Ankarafantsika National Park over a longer period of time (Wilmé et al., 2006). Coevolution of hosts and parasites over an extended period in the area may have led to higher immune competence of *M. ravelobensis* against filarial nematode species present in this area. Variations in MHC constitution, possibly due to demographic or pathogen-driven factors, have already been documented in *M. murinus* and sympatric congeners (*Microcebus griseorufus* and *Microcebus berthae*) in western and southeastern Madagascar (Sommer et al., 2014; Pechouskova et al., 2015). Furthermore, Schwensow et al. (2010) found a link between specific MHC alleles and resistance to intestinal nematodes in *M. murinus*. Differences in the genetic background, MHC diversity and possible correlations with disease risk in the two studied mouse lemur species require further investigation.

5. Conclusions

Both sympatric mouse lemur species, *M. murinus* and *M. ravelobensis*, are definitive hosts of filarial nematodes, but the risk of infection as well as microfilaremia differ between the two studied species. We did not observe a negative impact of filarial infections on host fitness, when taking body mass as a proxy, which may be explained by a persistent infection with low pathogenicity. The higher risk of infection and higher microfilaremia in *M. murinus* may be attributed to differing

immune competence between the two host species, possibly as a result of the more recent spatial expansion of the grey mouse lemur into northwestern Madagascar and a correspondingly shorter co-evolutionary history. Morphological characteristics of the microfilariae and adult specimen collected in this study compared to Ochoncercidae spp. previously described in lemurs suggest the presence of a new species, "*Lemurfilaria lemuris*". The detection of the same filarial parasite species in different hosts (*M. murinus*, *L. edwardsi*) indicates low host species-specificity. The obtained findings contribute to a better understanding of vector-borne diseases of Malagasy wildlife.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We would like to thank Djacoba Herrimann Tehindrazanarivelo, Eva Helene Kleine, Mona Zimmermann, Lotte Caecilia Striewe and Paul Tsiverizado for assistance during sampling and data collection in the field, as well as Ulla Küttler for assistance during laboratory procedures. We are grateful to the Malagasy government, Ministère De L'Environnement, des Eaux et Forêts and MNP for granting permission to work in the Ankarafantsika National Park (Autorisation de recherche N° 063/15/MEEMEF/SG/DGF/DCB.SAP/SCB du 12/03/2015, Autorisation de recherche N° 34/16/MEEMEF/SG/DGF/DAPT/SCBT.Re [Renouvellement de l'Aut N° 210/15 du 27/08/2015]) and greatly appreciate our long-term cooperation partners Solofonirina Rasoloharijaona, Blanchard Randrianambinina and Romule Rakotondravony from the University of Mahajanga for continuous support of our field work in Madagascar. This study was conducted under the Accord de Collaboration between the Institute of Zoology, Hanover, the University of Antananarivo and l'Association Nationale pour la Gestion des Aires Protégées (ANGAP, now Madagascar National Parks [MNP]).

The field research was supported by the Primate Conservation Inc., United States (Research Grant PCI# 1299), the German Academic Exchange Service, Germany (DAAD, travel grant no. 57212311) and the Gesellschaft für Primatologie, Germany (GfP, Christian-Vogel Fond). This publication was supported by Deutsche Forschungsgemeinschaft and University of Veterinary Medicine Hannover, Foundation within the funding programme Open Access Publishing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.07.003.

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