Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter now, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Lydia Rautman April 6, 2018

Effects of anthropogenic influence on patterns of infection with enteric zoonotic pathogens in brown mouse lemur, *Microcebus rufus,* at Ranomafana National Park, Madagascar

by

Lydia Rautman

Environmental Sciences

Thomas Gillespie, PhD

Adviser

Adrian Jaeggi, PhD

Committee Member

Carolyn Keogh, PhD

Committee Member

2018

Effects of anthropogenic influence on patterns of infection with enteric zoonotic pathogens in brown mouse lemur, *Microcebus rufus,* at Ranomafana National Park, Madagascar

By

Lydia Rautman

Thomas Gillespie, PhD

Adviser

An abstract of

a thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Arts with Honors

Environmental Sciences

2018

Abstract

Effects of anthropogenic influence on patterns of infection with enteric zoonotic pathogens in brown mouse lemur, *Microcebus rufus,* at Ranomafana National Park, Madagascar

By Lydia Rautman

Research on zoonotic transmission of enteric pathogens becomes increasingly relevant as anthropogenic influence brings humans and wildlife into close contact with one another. Previous studies have demonstrated higher rates of infection in primates in proximity to humans than those isolated from humans, but this study uniquely compares interfaces of lemurs with villagers and furthermore examines infection with multiple enteric pathogens. Outcome of infection in *Microcebus rufus*, the brown mouse lemur, was investigated as a result of type of anthropogenic influence in three study sites near Ranomafana National Park, Southeastern Madagascar. Brown mouse lemurs were trapped and sampled in a forest site (no human contact), near villages (high contact with villagers), and at a campsite (high contact with tourists and villagers). Over half (54.9%) of all lemurs sampled tested positive for at least one pathogen. Prevalence of infection was 11.1% at the forest site, 47.6% near the villages, and 81.0% at the campsite. Infection in *Microcebus* individuals was correlated with proximity to humans. Within the village study site, infection was geographically clustered. This study hypothesizes that introduction of novel pathogens could be a result of zoonotic transmission from nearby human populations. The results demonstrate the potential for zoonotic infection in a vulnerable species and highlight the need for further investigation as contact between humans and wildlife increases, increasing risk of infection for human and animal populations alike.

Effects of anthropogenic influence on patterns of infection with enteric zoonotic pathogens in brown mouse lemur, *Microcebus rufus,* at Ranomafana National Park, Madagascar

By

Lydia Rautman

Thomas Gillespie, PhD

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences

of Emory University in partial fulfillment

of the requirements of the degree of

Bachelor of Arts with Honors

Environmental Sciences

2018

Acknowledgements

Funding was generously provided by the Marcus Foundation, the Herrnstein Family Foundation, the Emory Global Health Institute, the Emory Scholars Program, and the Emory Master in Development Practice Program. I am grateful for logistical and infrastructural support from MICET, particularly director Dr. Benjamin Andriamihaja, and Centre ValBio, particularly director Dr. Patricia Wright. I am also thankful to the professors, students, post-docs, and staff who assisted on this project in both the laboratory at Emory University and in the field: Dr. John Wegner, Dr. Martin Kowalewski, Dr. Pascal Rabeson, Dr. Jean Claude Razafimahaimodison, Jean de Dieu Ramanantsoa, Maya Moore, Jesse McKinney, Telo Albert, Victor Tombotiana Aimé, Fidisoa Rasambainarivo, Kristen Cross, Emilie Redwood, Sarah Durry, Katherine Noble, Dr. DeAnna Bublitz, Leo Ragazzo, Kristin Derfus, Cassidy Rist, Caroline Schwaner, Dr. Sarah Zohdy, Dr. Ria Ghai, Christopher Wegner, and Rachel Kearns. Heritiana Anne Louisette, Rasolondraibe, Dr. Marissa Grossman, Ian Fried, Amelie Marcelle, Solo Justin, and François Zakamanana. This work could not have been completed without the support and expertise of my advisor, Dr. Thomas Gillespie, PhD, and the entire Gillespie lab; many thanks to my committee members, Dr. Adrian Jaeggi, and Dr. Carolyn Keogh.

Table of Contents

INTRODUCTION

Madagascar

Madagascar, just off the southeast coast of Africa, is an island brimming with biodiversity. Around 92% of its plant species and 85% of its animals and macroinvertebrates are endemic, providing an extremely diverse array of flora and fauna (Goodman & Benstead, 2005). Madagascar's varied topography creates a great number of different habitats, which allowed for early adaptive radiation of lemurs (Herrera, 2017). However, deforestation is major issue affecting these endemic inhabitants; one estimate places deforestation rates between 1.4-4.7% per year (Achard, Eva, Stibig, Mayaux, Gallego, Richards, & Malingreau, 2002). It is estimated that as much as 90% of the original vegetation has been lost (Ganzhorn, Lowry, Schatz, & Sommer, 2001; Tattersall, 2006). Furthermore, extensive deforestation like this enables humans to venture into previously undisturbed territory. This brings humans and wildlife into closer contact than ever before, presenting opportunities for transmission of pathogens and emergence of disease in new host species.

The country is also characterized by high rates of poverty; more than 91% of the population lives on less than $2.00/day (UNICEF, 2017). UNICEF has reported that the sanitation and hygiene conditions are the 4th lowest in the world and access to clean water is the 6th lowest (Government of Madagascar, 2016). In the rural villages, where some of the sampling for this study was done, open defecation rates are over 50% (Government of Madagascar, 2016). All of these factors result in the exacerbation of disease in local human populations, which can in turn lead to a greater exposure to enteric pathogens for the surrounding wildlife.

Zoonotic disease transmission

Research on zoonoses, pathogens able to be transmitted between humans and animals, is becoming an ever-more relevant field as humans engage in new interactions with wildlife and pathogens around the world. As over 60% of human infectious diseases have zoonotic potential, human encroachment on previously undisturbed habitat presents a health risk for wildlife species (Jones, Patel, Levy, Storeygard, Balk, Gittleman & Daszak, 2008). Especially at risk are primates, as their close phylogenetic relationship to humans results in higher potential for zoonotic transmission, as evidenced by the global HIV pandemic, Ebola outbreak, and others (Calvignac-Spencer, Leendertz, Gillespie & Leendertz, 2012). In many rural areas of the tropics, growing human populations and changes in land use increase overlap between humans and wildlife. This includes large-scale activities, such as extractive industries, or local small-scale interfaces, such as when humans venture into these areas in search of water or fuel wood. Conversely, increasingly fragmented habitats force animals to forage more widely for resources. These changes place people in closer, and many times more intimate, contact with wildlife, increasing zoonotic disease risk. Research has observed the impact of this alteration in patterns of land use change in the presence of human zoonotic pathogens in wild primates (Nunn & Gillespie 2015). Confirmed transmission of human pathogens to great apes has been reported with respiratory disease in chimpanzees in Cote d'Ivoire (Köndgen et al., 2008) and Uganda (Scully et al., 2018), as well as in mountain gorillas in Rwanda (Palacios et al., 2011). These patterns suggest a possible exposure to and sharing of zoonotic disease between humans and gorillas, and furthermore the likelihood of higher prevalence of pathogens with greater interactions with humans. As seen in these studies, much of the

research concerning human-primate disease transmission has been focused on the African apes, whose conservation has become an extremely political issue. However, research involving other endangered primates is just as crucial; this will provide a greater perspective on human-primate pathogen sharing and will enable researchers to assess transmission as a potential conservation concern.

Lemurs and *Microcebus rufus*

One such group of endangered primates is the lemurs. There are 97 extant species, of which 91% are classified as critically endangered, endangered, or vulnerable to extinction by the IUCN Red List (IUCN, 2017; Schwitzer et al., 2013). In the face of devastating deforestation and loss of biodiversity, lemurs are the prominent flagship species for conservation efforts in Madagascar. Recent scientific research has been investigating the impact of zoonotic disease on lemurs as a conservation concern.

This study examines the role of human contact in prevalence of zoonotic infection in the brown mouse lemur, *Microcebus rufus*. *M. rufus* was chosen as the study organism for a number of reasons; for one, its small size (approximately 40g) facilitates noninvasive sampling methods through trapping, compared to larger lemur species, which can weigh up to 9 kg. Its home range is around 0.5-1 hectare and it frequently overlaps with other mouse lemurs. *M. rufus* is primarily arboreal but also goes down to the ground, coming into contact with potentially contaminated water and soil. While some *Microcebus* species inhabit only one or two types of habitat, *M. rufus* is a generalist and has been found in a variety of different habitats with varying altitudes and rainfall. *M. rufus* has been previously noted for its abundance in and even preference for forest edges, which may be due to the availability of insect prey in those areas (Ganzhorn, 1995). This adaptability also brings

them in close contact with human communities, exposing them to zoonotic human pathogens through direct contact or the environment. Interactions with humans is likely to be more limited in other more specialist lemur species and thus our results will be an overestimate of pathogen prevalence in other lemurs. Still, our study of *M. rufus* may give insights about patterns of infection prevalence and proximity to human communities that are similarly observed in other lemurs.

Common zoonotic enteric pathogens in Madagascar

Four enteric pathogens were selected to be studied in *Microcebus rufus* due to their demonstrated high prevalence in human populations in Madagascar (Table 1). All four pathogens have been confirmed in both human and non-human primate species, indicating zoonotic potential. Furthermore, they cause diarrheal disease in humans and are spread fecal-orally through fecal matter-contaminated water, food, or soil. This transmission may potentially be exacerbated by Madagascar's poor sanitation infrastructure. *Shigella flexneri, Salmonella enterica,* and *Entamoeba histolytica* have been studied in mixed groups of lemurs, but have not focused on *Microcebus rufus* in particular (Bublitz, Wright, Rasambainarivo, Arrigo-Nelson, Bodager & Gillespie, 2015; Ragazzo, Zohdy, Velonabison, Herrera, Wright & Gillespie, 2018).

Shigella flexneri is a gram-negative bacterium causing shigellosis in humans and non-human primates (Jennison & Verma, 2004; Kennedy, Astbury, Needham, & Cheasty, 1993). *Shigella* is a major cause of childhood diarrheal disease in Africa and Asia (Liu et al., 2016) and prevalent in Madagascar (Giordano et al., 2018; Randremanana et al., 2012). In a 2015 study, *Shigella* was found with a prevalence ranging from 26.0-64.0% in three rural villages (Bublitz, Wright, Bodager, Rasambainarivo, Bliska & Gillespie, 2015). Another

study observed a prevalence of 22.0% along with *Shigella*-associated antibiotic resistance in the town of Ranomafana, Madagascar (Giordano et al., 2018). As antibiotics are often used in Madagascar without proper identification of the bacteria, over- and misuse of antibiotics has led to Ampicillin-resistant strains of both *Shigella* and *Salmonella* (Randrianirina et al., 2014). Additionally, a study of *Shigella* in lemurs in Ranomafana National Park near villages found a prevalence of 4.0% (Bublitz, et al, 2015).

Salmonella enterica is a bacterium causing enteric fever around the world (Zhang et al., 2003). It causes around 22 million cases a year, particularly where there is poor access to clean water and proper sanitation (Andrews-Polymenis, Bäumler, McCormick, & Fang, 2010). In the same study from 2015, *Salmonella* was found at a prevalence of 16.0-32.0% in the three villages and at 5.4% in Ranomafana. It was also found in lemurs with a prevalence of 8.0% (Bublitz, et al., 2015).

Giardia lamblia, also known as *Giardia intestinalis* and *Giardia duodenalis,* is a microscopic protozoan parasite that causes the diarrheal disease giardiasis in humans and other mammals (Adam, 2001). *Giardia* infections are found all over the world and are a leading cause of childhood morbidity and mortality where clean water and proper sanitation resources are lacking, as seen in Madagascar (Randremanana et al., 2012). In addition to its presence in humans, infection by *Giardia* has been recorded in wild non-human primates including lemurs (Ryan & Cacciò, 2013). Only one study has looked at *Giardia* in brown mouse lemurs; furthermore, this study had a much smaller sample size (4 *M. rufus* samples, 19 total lemur samples versus 51 *M. rufus* samples for this study). This study used immunofluorescence in identification of the pathogen instead of molecular methods, increasing the potential for false negatives; molecular methods are by

comparison much more precise in pathogen identification (Rasambainarivo et al., 2013; Thompson, 2004). Other similar studies have found *Giardia* in primate populations (Salzer et al., 2007) and due to its prevalence in humans in the region, *Giardia* is expected to be present in the brown mouse lemurs.

Entamoeba histolytica is a protozoan parasite causing the enteric disease amoebiasis (WHO, 2018). Infection is most commonly caused by contaminated water or food (Ravdin, 1989). It is most common in developing countries in the tropics (WHO, 2018) and has been found in humans and non-human primates (Legesse & Erko, 2004; Smith & Meerovitch, 1985). The infection has been found in humans in Madagascar (Randremanana et al., 2012) and a recent study found it in *Microcebus rufus* at a 5.4% prevalence (Ragazzo et al., 2018).

The examination of both bacterial and protozoal pathogens is important as the two groups can differ biologically in ways that could affect transmission rates. Protozoa are extremely hardy and can persist in the environment for extended periods of time; *Giardia lamblia* and *Entamoeba histolytica* can persist in cysts in soil for a week or in water for several weeks (CDC, 2015; Petri & Singh, 1999). While bacterial diseases are easier to treat, high rates of antibiotic resistance have been observed in Madagascar, making the diseases a persistent issue (Randrianirina et al., 2014).

Anthropogenic influence and disease in lemurs

Recent studies have demonstrated transmission of human pathogens to wild primates, especially near tourism and research (Köndgen et al., 2008; Palacios et al., 2011; Scully et al., 2018). Similarly, research on enteric pathogens in humans and lemurs near Ranomafana National Park has demonstrated that lemurs exhibit higher prevalence of enteric pathogens in areas where they overlap with humans. In one study, the prevalence

of enterotoxogenic *E. coli* infection in lemurs was much higher in disturbed (overlapping with humans) than undisturbed (isolated) habitat at 61.0% versus 0.0% (Bublitz et al., 2015). Other studies found a Norovirus GII prevalence of 27.0% in lemurs near villages (Zohdy et al., 2015), and a difference in prevalence of *Entamoeba histolytica* between disturbed and undisturbed habitats at 4.7-14.3% versus 0.0% (Ragazzo et al., 2018). Now, my study will examine how presence and prevalence of enteric pathogens in brown mouse lemurs is correlated with type of anthropogenic influence.

Past studies have been instrumental in opening up the field to scientific inquiry about enteric pathogen transmission, but many questions need further investigation. While past research has only looked at the difference of infection in undisturbed versus disturbed habitat, this study is unique in its comparison of infection prevalence in the lemurs at village sites and those near the tourism site. Extremely high tourism rates will result in more frequent contact between humans and mouse lemurs; we expect to see a higher prevalence of infection at the campsite than at any other site. Although interactions between humans and lemurs are much less frequent at the village sites as they coexist peacefully, the high rates of human disease in the villages prompt us to expect higher prevalence of infection at the village sites than at the forest site. Results consistent with the hypotheses could suggest a link between type or amount of human activity and impact on lemur populations. Furthermore, the data from this study will contribute to the current body of information on lemur-human disease transmission and will call for more attention to the issue as a conservation concern for other lemur species.

Study objectives and hypotheses

Microcebus rufus individuals were sampled in two study years in Southeastern Madagascar. In 2011, lemurs were sampled at the Centre ValBio campsite and at the Valohoaka forest site. In 2017, lemurs were sampled near villages within 5 kilometers of Ranomafana National Park. Samples were compared across the three sites in terms of anthropogenic influence: forest (low human contact), villages (high contact with villagers), and campsite (high contact with tourism-associated humans, including tourists, researchers, and field guides). The temporal difference between sample collections could have a minor influence on results, but this study assumes little year-to-year variation.

All samples were tested for four enteric pathogens that had previously demonstrated zoonotic transmission potential: *Salmonella enterica, Shigella flexneri, Entamoeba histolytica,* and *Giardia lamblia.* The objective of the study was to compare the presence and prevalence of these enteric diseases across three sites with different types of anthropogenic influence.

My hypotheses are as follows:

1) Infection of mouse lemurs by enteric zoonotic pathogens will only be present in areas of high human contact (village sites and campsite).
2) Prevalence of infection with any pathogen will be higher at the campsite than at the village sites; prevalence of infection with any pathogen will be higher at the village sites than at the forest site. Therefore, the order of prevalence from lowest to highest will be: forest, villages, campsite.

METHODS

Ethics statement

The IACUC protocol #3000417 "Identifying Risk Factors Associated with Diarrheal Disease in Rural Madagascar" for this research was reviewed and approved by the Institutional Animal Care and Use Committee; all primates were treated ethically during the course of this research.

Field Methods

Study Site

The Ifanadiana district (21°18′S 47°38′E) in southeastern Madagascar is the focus of long-term interdisciplinary research involving the Gillespie lab group in partnership with the Centre ValBio (CVB) research station, Harvard Medical School, Madagascar's Ministry of Health (MOH), and PIVOT, an organization dedicated to improving health care in the region. The human population within this district suffers from extreme poverty, infectious disease and mortality rates that exceed national averages, and experience unique interfaces with the wildlife of Ranomafana National Park.

The Valohoaka forest site is densely forested and difficult to access because of its location several kilometers away from the main road (21° 17.884 S, 47° 26.373 E). The campsite is located close to Centre ValBio research station and near the resort town of Ranomafana (Figure 1). The site is on the national highway, the only paved road in the region, so it sees heavy traffic from both international and local tourists. The human activity at this tourist site is concentrated in a couple of months of the year, increasing opportunity for human-lemur contact. There are latrines at this site and tourists accustomed to modern toilets are more likely to use them than defecate in the forest. Although not allowed, forest

guides sometimes bait lemurs to come down to the ground to interact with tourists, and the lemurs, as a result, are more habituated to human presence (P. Wright, Personal Communication).. This change in behavior brings them into more frequent contact with potentially contaminated soil and water, increasing exposure to zoonotic pathogens.

The village sites are distinctly different from the campsite; the rural villages are in roadless areas that can be 2-8 hours hiking from the nearest road. The villages are subsistence agricultural communities and participate only minimally in the cash economy. The villagers grow their crops in the rice paddies located outside the villages and interact primarily with others within the village or in nearby villages; only occasionally do they leave to visit a larger town like Ranomafana, usually on market day. As a result of intervention efforts, there are latrines in many of the villages but they are not often used; open defecation rates in the villages remain at over 50% (Government of Madagascar, 2016). Diarrheal disease is a leading cause of mortality in Madagascar (Marks et al., 2016). As observed in other African countries, the lack of accessible healthcare and clean water in addition to behavioral factors like open defecation results in higher diarrheal disease prevalence in rural than urban areas (Jamison, 2006). The porous boundary of Ranomafana National Park allows for a unique interface between the villagers and the surrounding wildlife: villagers may enter the forest and lemurs or other animals may leave the forest, both of which increase risk of exposure to zoonotic pathogens. However, the villagers are more accustomed to the presence of these lemurs than are tourists, and villagers and brown mouse lemurs coexist peacefully.

Between May and August 2017, members of the Gillespie lab traveled to Madagascar and worked with a local health team to visit eight villages located within 5 kilometers of

Ranomafana National Park. There, *Microcebus rufus* individuals were trapped near the villages and sampled, but data was only collected in five of these villages (Figure 1). Data was collected in vegetation adjacent to footpaths where it was believed there was the greatest overlap between lemurs and villagers (Bodager et al. 2015; Bublitz et al. 2015). In 2011, Gillespie lab researchers travelled to Madagascar and collected data from seven species of lemurs. *Microcebus rufus* individuals were captured at two sites with differing types of anthropogenic influence: forest and campsite.

Trapping and sample collection

In both study years, mouse lemurs were captured using banana-baited Sherman traps along footpaths at the study site (XLR, Sherman Traps Inc., FL). In order to sample lemurs living closest to the villages, the main footpath outside the village was used as the transect and traps were set at ~10-meter intervals, 1-3 meters off the transect (alternating off the right and left sides of the path), and 1-2 meters off of the ground (Figure 2). A total of 25-30 or more traps were set each night along a single transect, extending the line of traps for 250-300+ meters. Over three months in 2017, a total of more than 700 traps were set at the village sites. A slice of banana was rubbed on the sides and top of the trap to attract the lemurs by scent and the remainder of the slice was placed in the back of the trap. Trap setting methods used at the campsite and forest site were similar to the approach taken in the 2017 sample collection period but traps were set at ~50m intervals. As *Microcebus rufus* is a nocturnal species (Radespiel, 2006), traps were set at around 16:00 and collected at 05:00.

At the forest site, *Microcebus* population densities were lowest and traps were checked at 20:00 and 05:00 to increase capture success. At the campsite, traps were checked at 20:00; at the village sites, traps were checked at 05:00. A total of 21 lemurs

were caught at the village sites, 21 at the campsite, and 9 at the forest site. When a lemur was caught in a trap, it was released into a plastic Ziplock bag and weighed using a spring scale (Eisco labs 100g spring balance); the weight of the plastic bag was later subtracted from the total weight. The lemur was removed by gloved hand and was carefully extended to collect measurements (Figure 3). Sex was recorded, as were notes about general health and appearance. Photographs were taken of each lemur and individuals were marked with a permanent marker on the base of the tail to enable detection of recapture. The lemur was released on the branch on which it was captured and GPS coordinates were taken from that spot. Finally, the trap was opened up to facilitate the collection of a 1mL fecal sample with a sterile tongue depressor, which was then transferred into a cryovial filled with approximately 0.8mL RNAlater and barcoded for inventory. Traps were re-baited for two or three nights and recapture was ruled out with markings and unique measurements of individuals. Traps that had contained a rat or lemur were brought back to the village for cleaning and replaced in the evening, when banana in the traps was also replaced. When a new path was selected, it was farther than 100 meters from the previous path to reduce risk of recapture.

Lab methods

DNA extraction

DNA from the 2017 village samples was extracted at the Centre ValBio research station using ZymoBIOMICS™ DNA Miniprep Kits and following instruction manual protocol. DNA extraction protocol was the same for the 2011 samples but used the FastDNA SPIN Kit for Soil (MP Biomedicals, LLC, Solon, OH) and followed protocols from Gomes et al. (1999). Both DNA extraction processes consisted of the following:

Fecal samples preserved in RNAlater were vortexed to homogenize the solution. Of this solution, 250mL was added to BashingBeads™ and 750µl Lysis Solution was added. The fecal DNA and Lysis solution was vortexed using the Genie 2™ cell disruptor for 15 minutes, then was centrifuged at 10,000 x *g*. The supernatant was transferred to the Spin Filter, centrifuged through, and 1,200µl Binding Buffer was added to the filtrate. Approximately 800µl of this mixture was transferred to the IIC Column filter and centrifuged through, the flow through discarded, and the rest of the mixture was filtered through the same filter. The DNA Wash Buffer 1 was added to this Column Filter and centrifuged through, then the DNA Wash Buffer 2 was filtered through twice. DNase/RNase Free Water was filtered through to elute the DNA; the DNA was filtered through the Spin Filter and into a clean microcentrifuge tube.

PCR and gel electrophoresis

All pathogen testing was conducted in the Emory University lab. Polymerase Chain Reaction (PCR) was conducted on all samples to amplify the DNA along the sequences of base pairs that matched the primers, which worked as an identifier for a pathogen (Table 3). Samples were run in the thermocycler for cycles at specific temperatures that separated the DNA strands and selectively amplified the targeted DNA. *E. histolytica* was tested for with the small subunit rRNA gene (GenBank accession no. X64142) (Foo et al., 2012); the invasion protein invA was targeted for *Salmonella*; and the invasion plasmid antigen H (ipaH) gene was targeted for *Shigella.* Because *Giardia* genotypes are so morphologically similar, a multi-locus molecular approach was required to identify the genotypes and reduce the risk of false negatives (Feng & Xiao, 2011). PCRs for *Giardia* loci were nested, requiring two rounds of PCR. Samples were tested for *Giardia* at three different loci*:* triose-

phosphate isomerase (tpi), glutamate dehydrogenase (gdh), and beta-giardin (bg). Tpi and gdh are housekeeping enzymes, while bg is a protein specific to *Giardia lamblia.* Molecular testing for all three genes is necessary to ensure accuracy of prevalence estimates and a positive result for any of the three genes indicates the presence of *Giardia lamblia.*

Gel electrophoresis was used to visualize which samples were positive for the pathogen: 5µl of PCR product was stained with 1ul of blue loading dye and pipeted into wells formed in a 1.6% agarose gel. An electric field was applied and the molecules were run through the gel matrix at different speeds according to the weight of the DNA fragment, determined by the number of base pairs. The bands produced from this phosphoresced under UV light and were run against a DNA ladder for reference of band size. Bands at the product length of the targeted pathogen (Table 4) indicated a positive result.

Statistical Methods

All statistical analysis was done with R Studio, version 1.1.383, RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL . A general logistic regression model was used to determine the relative odds of infection at the three sites: forest, village, and campsite. This model and a multiple comparison test (multcomp package in R) were used to compare the three sites to each other in pairs. Effects of sex and condition of the mouse lemurs on infection were also examined. Condition was determined using the formula (weight/length from nose to tip of tail), as we expected this to be a good indicator of health. 16 lemurs from the village sites were used in this analysis, as this data was not available for the 2011 samples and for five of the 2017 samples.

Use of data

Some samples used for analysis were collected in a separate study in 2011 by an interdisciplinary team led by Dr. Gillespie including Deanna Bublitz, Fidy Rasambainarivo and Emo Redwood. Sarah Zohdy joined the lab in 2012 and oversaw the PCR analyses of the samples by Ian Fried, Marissa Grossman and Kristen Cross, and Leo Ragazzo.

RESULTS

A total of 51 *Microcebus rufus* were caught and sampled (Table 2). Nine individuals were sampled from core forest habitat (no regular overlap with humans) and 21 were sampled from a campsite (overlap with tourists and villagers) in 2011. Twenty-one were sampled from forest edge adjacent to villages (overlap with villagers) in 2017. Of the 51 individuals, 28 (54.9%) tested positive for at least one enteric pathogen. Of the lemurs infected with at least one pathogen, *Giardia* was the most common (100% of infected lemurs, N=28), followed by *Entamoeba histolytica* (10.7%, N=3), and then *Salmonella enterica* (3.6%, N=1) (Table 4). *Entamoeba* and *Salmonella* were geographically distinct; all *Entamoeba* positives were found in "campsite" lemurs and the single case of *Salmonella* was in a "village" lemur. In total, four of the 28 infected lemurs were positive for two pathogens (14.3%): one of these was in the village habitat and three were from the campsite. No lemurs tested positive for *Shigella* and no lemur was infected with more than two pathogens. Infection was observed at all three sites; overall prevalence varied by site with forest lemurs having the lowest prevalence (11.1%, N=1/9), followed by village lemurs (47.6%, 10/21), and finally the campsite had the highest prevalence (81.0%, 17/21) (Figure 4). Mean infection prevalence by any pathogen was 54.9%.

A general logistic regression model was used to determine relative odds of infection across sites. Infection was 34.0x more likely at the campsite than at the forest site; 7.3x more likely at the village site than the forest site; and 4.7x more likely at the campsite than at the village sites. Infection prevalence was higher at the campsite than at the village sites (P=0.029) and was also higher at the campsite than at the forest site (P=0.003), supporting Hypothesis #2 (Table 5). A comparison of forest versus village sites was not significant

using a two-tailed test (P=0.084); however, as we expected directionality between the sites, we also conducted a one-tailed test, which was significant (P=0.042). One-tailed tests of the other two site comparisons were also significant (village-campsite P=0.015, forest-campsite P=0.002).

In our analysis of effect of sex or condition on infection, neither variable was found to have a significant effect on infection (Table 6) (sex: P=0.800; condition: P=0.261).

2017 Subset

In the 2017 lemur subset, eight villages were sampled but the total 21 lemurs came from only five village sites. Excluding villages in which no lemurs were captured, number of lemurs captured per village ranged from one to nine (4.2 on average). In one village, prevalence of disease in lemurs was 0.0% (Mangevo; N=5), and in two villages prevalence was 100% (Bevoahazo, Beremby; N=3, 1). In the remaining two villages prevalence was 33.3% (Ambinandranfotoka; N=3) and 56.6% (Miaranony; N=9) (Figure 5).

DISCUSSION

Zoonoses and *Microcebus*

Zoonoses, pathogens that can be transmitted between species, are one of the most important and groundbreaking areas of research in fields of public health and conservation today. Especially at risk are wild primates like lemurs because they are phylogenetically closer to *Homo sapiens* than any other mammal (Brack, 1987; Wolfe et al., 1998). More recently, conservation research has sought to determine the role of humans in the endangerment of lemur species through the sharing of diseases. Several studies take a special interest in *Microcebus rufus*, the brown mouse lemur, as a potential host of enteric pathogens due to its nature as a habitat generalist, abundance in forest edges, and frequent interaction with humans (Ganzhorn, 1995; Lehman, Rajaonson, & Day, 2006; Radespiel, 2006).

This study investigates disease transmission between lemurs and humans as influenced by type of exposure to humans. Previous studies in the Gillespie Lab have demonstrated that lemurs exhibit higher prevalence of enteric pathogens where they interface with humans (Bublitz et al., 2015; Ragazzo et al., 2018). These studies also pointed to several specific pathogens with the potential for zoonotic transmission (Table 1). The current study builds on these previous efforts and contributes the novel examination of lemurs overlapping with rural human populations in roadless areas without tourism. The subsistence agriculturalists from the village sites are socioeconomically and behaviorally different from those at the campsite, which includes local humans engaged in the cash economy along the road and tourists. This may provide insights about whether there is a

difference in infection prevalence between the types of human exposure or if it is any general human contact that most greatly influences disease in lemurs.

This study examined zoonotic pathogen prevalence in *Microcebus rufus* in three different habitats because of its frequent interface with humans. Infection prevalence of any pathogen was at its highest prevalence at the campsite, and second-highest at the village sites, and lowest at the forest site.

Infection presence and prevalence

Infection was observed in at least one individual at all three sites, refuting Hypothesis #1. *Giardia* was much more prevalent than any other pathogen and was found at all three sites. In contrast, the other pathogens were geographically concentrated at the sites: *Salmonella* was only observed in the village sites and *Entamoeba* was only present at the campsite. In the context of pathogen endemism research (Hudson, Rizzoli, Grenfell, Heesterbeek & Dobson, 2002), the presence of *Giardia* even in isolated habitat and its high prevalence in comparison with the other pathogens leads us to hypothesize that *Giardia* may in fact be enzootic to these lemur populations. On the other hand, *Salmonella* and *Entamoeba histolytica* were found at a much lower prevalence and are more likely to be novel pathogens, the low prevalence the result of a single incidence of exposure. It is possible that novel pathogens cause more serious sickness in mouse lemurs; if diseased, mouse lemurs are less active and this could lower their chances of being sampled, making our estimates for novel pathogen prevalence more conservative than the actual prevalence.

Other external factors that could have influenced the prevalence observed must be considered. For instance, the tropical cyclone Enawo hit Madagascar in March 2017 and lemurs sampled near the villages could have been impacted by it. Higher-than-usual rates

of runoff could have the potential to either mitigate disease spread by "cleaning" the area; however, they could also exacerbate it by facilitating transmission pathways, for example by flooding latrines and washing the contents into the forests. Further research is needed to eliminate temporal biases between study groups by sampling all individuals in the same year and season.

Site comparisons

A comparison of prevalence between the forest and village sites resulted in a P-value of 0.084 with a two-tailed test (Table 5). However, the observed prevalence at the village sites was clearly higher than at the forest site (47.6% versus 11.1%), and a small sample size is likely the cause of the insignificant P-value. Because we know that human contact is higher at the village sites than at the forest site, we predicted directionality and also conducted a one-tailed test, for which the P-value was 0.042. Further research with a larger sample size will allow for a more definitive statistical analysis. Acknowledging this, we found our prevalence ranking from lowest to highest to be forest, village, and campsite, supporting Hypothesis #2.

Prevalence of infection was significantly higher at the campsite, for which there are a number of possible explanations. One possibility is the difference in types of human activity. Human activity is much greater at the campsite due to the concentration of tourism during a couple of months of the year. This tourism has likely altered the behavior of the lemurs themselves, habituating them to the presence of tourists and making them more likely to come down to the ground, increasing exposure to diarrheal pathogens. This is different from at the village sites, where humans and lemurs coexist without much forced interaction between the two groups; lemurs probably come down to the ground less

frequently, decreasing exposure to fecal matter-contaminated soil. The high density of humans and lemurs at the campsite also increases risk of reinfection, particularly with *Giardia,* which has the potential to reinfect a host after treatment or recovery (Gilman, Miranda, Marquis, Vestegui & Martinez, 2003). Stress from habitat alteration and presence of humans could also make campsite lemurs more susceptible to infection. As studied in humans and other primates, chronic stress can suppress immune function by decreasing immune cell numbers and function (Dhabhar, 2009); stress from human-associated disturbances could certainly have an impact on immunological health in lemurs. The high densities of the mouse lemurs at the campsite could also cause stress and likely result in more frequent and inter- and intra-species contact, increasing opportunities for pathogen transmission (Lafferty & Gerber, 2002).

Infection prevalence was moderate at the village sites, where lemurs are exposed solely to local populations of humans, as compared to in the forest habitat. The interactions of these lemurs with humans are most likely in the form of indirect contact, for example through environmental contamination. The high rates of infection at the village sites are likely explained by the high rates of disease in humans at these sites, which would theoretically increase the mouse lemurs' exposure to these pathogens. Open defecation behavior in the villages may result in environmental contamination; even where latrines are used, flooding during the rainy season can cause latrine contents to be spread to the nearby forest habitats of lemurs. The proximity of the national park to the villages allows for frequent indirect contact between humans and the reserve that is not present in other areas. Rural human populations often enter the forest to extract resources such as firewood and food, but may also defecate in the forest; meanwhile, animals from the forest may enter

the villages to raid village crops, leaving behind their own droppings. Both of these scenarios provide more opportunities for interaction, both direct and indirect, between lemurs and humans. Further analyses are underway to determine whether or not humans are actually a source of the *Giardia* observed in these populations. If the sequence results don't suggest humans as vectors for this disease, high prevalence in village-associated lemurs could be explained instead by high population densities of lemurs near forest edges and associated frequent intraspecies contact. Other organisms like cows, dogs, and pigs could also be vectors for the pathogen, as they spend considerable time in the vegetation close to the villages, rooting for food and defecating.

Comparison of human and lemur pathogens

Curiously, we found no evidence of *Shigella* in the lemurs sampled; this may suggest either a lack of *Shigella* in the nearby human populations or could indicate that transmission is more "difficult" for this pathogen, despite the similar fecal-oral transmission route. Preliminary results of humans sampled simultaneously for *Shigella* in the same villages demonstrated a prevalence of 38.2% (Gillespie et al., unpublished data), which provides support for the latter hypothesis and encourages us to consider why a pathogen with zoonotic potential and ample opportunity may not be transmitted. One may find that some enteric pathogens are more easily shared ("more zoonotic") while others less so, as they are expected to all be shared by approximately the same mechanisms. Different types of pathogens also differ in biology; protozoal pathogens like *Giardia* can also exist in an inactive form called a cyst, which can persist in the environment for up to a few weeks (CDC, 2015). This makes protozoa much more environmentally persistent than bacteria, perhaps explaining why a much higher prevalence of *Giardia* was observed than

the bacterial pathogens. Furthermore, both *Giardia* and *Entamoeba* are most commonly asymptomatic, in which state they produce the environmentally-resistant cysts, making asymptomatic infection the most effective form for transmission (Gardner & Hill, 2001; WHO, 1997). However, this does not account for why prevalence of *Giardia* was so much greater than that of *Entamoeba*. Future studies should directly compare the infection profile of a human community to its adjacent lemur population to see how similar they are in pathogen makeup and prevalence. The nature of a study conducted in the wild makes it difficult to parse out which elements are having the observed effect. Especially where there is high human-lemur contact, there are many different modes of transmission occurring and it is challenging to determine exactly what interactions increase risk of disease. Modes of transmission should be further investigated to determine through which route there is the most pathogen sharing; such information would facilitate efforts to mitigate disease sharing between humans and lemurs.

Other analyses

We did not find sex or condition of lemur to be correlated with infection; this indicates that females do not differ from males in likelihood of infection, although a larger sample size would help to confirm this hypothesis. Condition may not predict infection because the pathogen does not negatively impact the host; this is further supporting by our observations of infected lemurs being asymptomatic.

Interestingly, we found a trend in the geographical analysis of prevalence at the different village sites. The two villages with the highest prevalence of infection, Bevoahazo and Beremby (100% infection prevalence at both) are only about 5 kilometers away from each other (Figure 5). The two villages with the lowest prevalence of infection,

Ambinandranfotoka and Mangevo (33.3% and 0.0% respectively) are only a couple of kilometers away from each other, but the two groups of villages are separated by about 10 kilometers, including a section of Ranomafana National Park. It is possible that the high flux of humans between villages located near each other would cause similar rates of disease among those villages, whereas the villagers would be much less likely to visit another village more than 10 kilometers away. This could result in locally similar rates of endemism within village clusters that vary between groups of villages; further research with a larger number of samples is needed to determine this possible connection.

Summary of discussion

Prevalence at the campsite was significantly higher than at the village sites, and prevalence at the villages was higher than at the forest sites, suggesting that high human activity increased risk of infection. A significantly different prevalence at the campsite compared to the village sites suggest that the different human-lemur interfaces pose different levels of risk of infection, which could be a result of differences in exposure or susceptibility of the lemurs themselves. In conjunction with other literature, this study provides more support for the hypothesis that lemurs living in proximity to humans, particularly in areas associated with tourism, may be at greater risk for infection with zoonotic disease. This may suggest that some sharing of pathogens is occurring, whether by direct contact or through environmental contamination. Interestingly, we found that among village-associated lemurs, prevalence may be clustered in specific regions, possibly explained by the flux of humans between villages near each other.

Future research

Further study of pathogen transmission in this ecosystem is necessary to understand the possible sharing pathways between lemurs and humans. Ideally, another study would be able to address the torpor issue that likely limited the catch success of this study; due to academic scheduling, samples were only able to be collected during the Austral winter, when mouse lemurs were experiencing torpor. Sampling in the months of September and October would yield many more samples, bolstering the viability of the statistical analyses. Similar studies should be conducted with other lemur species to see if the pattern of higher infection prevalence near tourism sites holds true; furthermore, if moderate infection rates near local communities is also reflected in other species. If these patterns are present in other lemur species, this may help with the development of human-lemur disease transmission theories that could be utilized in biodiversity conservation efforts on the island.

Conclusion

This study is significant in its comparison of different types of lemur-human interfaces and the resulting pathogen prevalence. For the first time, infection in lemur populations in a tourism-associated habitat was contrasted against those living near villages. Information from this study and similar research could suggest potential transmission pathways that put endangered lemur communities at risk. The ability to identify such pathways enables conservation efforts to better target efforts and implement interventions, such as community education in the villages or at tourist sites. This work seeks to highlight the necessity of investigating zoonotic disease as a conservation concern for primates around the world.

Future research is critical to providing support to the hypothesis that pathogens are being shared between human and wildlife communities. Zoonotic disease transmission will continue to be a field critical to both wildlife conservation and global health research. As humans continue to harvest natural resources and facilitate tourism, human-wildlife interactions will increase in frequency. Such changes could allow for the spillback of or emergence of new infectious diseases, with serious implications for animal and human populations alike.

REFERENCES

Achard, F., Eva, H. D., Stibig, H. J., Mayaux, P., Gallego, J., Richards, T., & Malingreau, J. P. (2002). Determination of deforestation rates of the world's humid tropical forests. *Science*, *297*(5583), 999-1002.

Adam, R. D. (2001). Biology of *Giardia lamblia*. *Clinical microbiology reviews, 14*(3), 447-475.

Andrews-Polymenis, H. L., Bäumler, A. J., McCormick, B. A., & Fang, F. C. (2010). Taming the elephant: *Salmonella* biology, pathogenesis, and prevention. *Infection and immunity, 78*(6), 2356-2369.

Brack, M. (1987). Viruses. In M. Brack, Agents Transmissible from Simians to Man (pp. 1-95). Berlin, Heidelberg: Springer Berlin Heidelberg.

Bublitz, D. C., Wright, P. C., Bodager, J. R., Rasambainarivo, F. T., Bliska, J. B., & Gillespie, T. R. (2015). Epidemiology of pathogenic enterobacteria in humans, livestock, and peridomestic rodents in rural Madagascar. *PloS one, 9*(7), e101456.

Bublitz, D. C., Wright, P. C., Rasambainarivo, F. T., Arrigo-Nelson, S. J., Bodager, J. R., & Gillespie, T. R. (2015). Pathogenic enterobacteria in lemurs associated with anthropogenic disturbance. *American Journal of Primatology, 77*(3), 330-337.

Calvignac-Spencer, S., Leendertz, S.A., GIllespie, T.R., Leendertz, F.H. (2012). Wild great apes as sentinels and sources of infectious disease. *Clinical Microbiology and Infection*, *18*(6): 521-7.

Centers for Disease Control (CDC). (2015). Giardia & pets. Atlanta, Georgia, USA.

Dhabhar, F. S. (2009). Enhancing versus suppressive effects of stress on immune function: implications for immunoprotection and immunopathology. *Neuroimmunomodulation*, *16*(5), 300-317.

Dobson, A., & Foufopoulos, J. (2001). Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society of London B: Biological Sciences, 356*(1411), 1001-1012.

Feng, Y. & Xiao, L. (2011). Zoonotic Potential and Molecular Epidemiology of *Giardia* Species and Giardiasis. *Clinical Microbiology Reviews, 24*(1), 110-140.

Foo, P.C., Chan, Y.Y., Too, E.C.S., Tan, Z.N., Wong, W.K., Lalitha, P. & Lim, B.H. (2012). Development of a thermostabilized, one-step, nested, tetraplex PCR assay for simultaneous identification and differentiation of *Entamoeba* species, *Entamoeba histolytica* and *Entamoeba dispar* from stool samples. *Journal of Medical Microbiology, 61*(9), 1219-1225.

Ganzhorn, J. U., Lowry, P. P., Schatz, G. E., & Sommer, S. (2001). The biodiversity of Madagascar: one of the world's hottest hotspots on its way out. *Oryx*, *35*(4), 346-348.

Ganzhorn, J. U. (1995). Low-level forest disturbance effects on primary production, leaf chemistry, and lemur populations. *Ecology, 76*(7), 2084-2096.

Gardner, T. B., & Hill, D. R. (2001). Treatment of Giardiasis. *Clinical Microbiology Reviews*, *14*(1), 114–128. http://doi.org/10.1128/CMR.14.1.114-128.2001

Gilman, R.H., Miranda, E., Marquis, G.S., Vestegui, M., Martinez, H. (2003). Rapid reinfection by Giardia lamblia after treatment in a hyperendemic third world community. *The Lancet, 331*(8581), 343-345.

Giordano, R. C., Rist, C.L., Ghai, R.R., Parsons, M.B., Narharimehefa, A., Bridges… Gillespie, T.R. (2018). High Rates of Shigella-Associated Antibiotic Resistance in Rural, Forested Madagascar. *In review.*

Gomes, M.A., Pequero, J.B., Furst, C., Valle, O.R., Pequero, J.L, Silva, E.F. (1999). An improved method to distinguish *Entamoeba histolytica* and *Entamoeba dispar*. *Parasitology, 119*(4), 359-362.

Goodman, S. M., & Benstead, J. P. (2003). Natural history of Madagascar. Chicago, IL: University of Chicago Press.

Goodman, S. M., & Benstead, J. P. (2005). Updated estimates of biotic diversity and endemism for Madagascar. *Oryx, 39*(1), 73-77.

Government of Madagascar: Ministry of Water, Sanitation. and Hygiene (2016). Investing in Water, Sanitation and Hygiene in Madagascar: the Business Case. Antananarivo, Madagascar.

Herrera, J. P. (2017). Testing the adaptive radiation hypothesis for the lemurs of Madagascar. *Royal Society Open Science, 4*(1).

Hudson, P.J., Rizzoli, A., Grenfell, B.T., Heesterbeek, H., & Dobson, A.P. (Eds). (2002). The Ecology of Wildlife Diseases (1st ed.). Oxford, UK: Oxford University Press.

Innes, J. L. (2010). Madagascar rosewood, illegal logging and the tropical timber trade. *Madagascar Conservation & Development, 5*(1).

IUCN, International Union for Conservation of Nature (2017). T*he IUCN Red List of Threatened Species* (Version 2017-3). Cambridge, United Kingdom.

Jamison, D. T. (Ed.). (2006). *Disease and mortality in sub-Saharan Africa*. World Bank Publications.

Jennison, A. V., & Verma, N. K. (2004). *Shigella flexneri* infection: pathogenesis and vaccine development. *FEMS microbiology reviews, 28*(1), 43-58.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, *451*(7181), 990-3.

Kennedy, F. M., Astbury, J., Needham, J., & Cheasty, T. (1993). Shigellosis due to occupational contact with non-human primates. *Epidemiology & Infection, 110*(2), 247-251.

Köndgen, S., Kühl, H., N'Goran, P.K., Walsh, P.d., Schenk, S., Ernst, N., ...Leendertz, F.H. (2008). Pandemic human ciruses cause decline of endangered great apes. *Current Biology, 18*(4), 260-264.

Lafferty, K. D., & Gerber, L. R. (2002). Good medicine for conservation biology: the intersection of epidemiology and conservation theory. *Conservation Biology, 16*(3), 593-604.

Legesse, M., & Erko, B. (2004). Zoonotic intestinal parasites in Papio anubis (baboon) and Cercopithecus aethiops (vervet) from four localities in Ethiopia. *Acta Tropica, 90*(3), 231-236.

Lehman, S. M., Rajaonson, A., & Day, S. (2006). Edge effects and their influence on lemur density and distribution in southeast Madagascar. *American Journal of Physical Anthropology, 129*(2), 232-241.

Liu, J., Platts-Mills, J. A., Juma, J., Kabir, F., Nkeze, J., Okoi, C., …Houpt, E.R. (2016). Use of quantitative molecular diagnostic methods to identify causes of diarrhoea in children: a reanalysis of the GEMS case-control study. *The Lancet, 388*(10051), 1291-1301.

Marks, F., Rabehanta, N., Baker, S., Panzner, U., Park, S.E., Fobil, J.N., … Rakotozandrindrainy, R. (2016). A way forward for healthcare in Madagascar? *Clinical Infectious Diseases, 62*(S1), S76-79.

Nelson, R. J. (2004). Seasonal immune function and sickness responses. *Trends in immunology, 25*(4), 187-192.

Nunn, C., Gillespie, T.R. (2016). Pathogens and primate conservation. In S. Wich & A. Marshall (Eds.), Primate Conservation (pp. 157-174). Oxford, UK: Oxford University Press.

Palacios, G., Lowenstine, L.J., Cranfield, M.R., Gilardi, K.V.K., Spelman, L., Lukasik-Braum, M., …Lipkin, W.I. (2011). Human metapneumovirus infection in wild mountain gorillas, Rwanda. *Emerging Infectious Diseases*. *17*(4), 711-713.

Parsons, M.B., Travis, D., Lonsdorf, E.V., Lipende, I., Roellig, D.M.A., Kamenya, S.,… Gillespie, T.R. (2015). Epidemiology and molecular characterization of *Crytosporidium* spp. in humans, wild primates, and domesticated animals in the greater Gombe ecosystem, Tanzania. *PLoS Neglected Tropical Diseases 9*(3), e0003529.

Petri, W.A., & Singh, U. (1999). Diagnosis and management of Amebiasis. *Clinical Infectious Diseases 29*(5), 1117-1125.

Radespiel, U. (2006). Ecological diversity and seasonal adaptations of mouse lemurs (*Microcebus* spp.). *Lemurs: ecology and adaptation. Springer, New York*, 211-233.

Ragazzo, L. J., Zohdy, S., Velonabison, M., Herrera, J., Wright, P. C., & Gillespie, T. R. (2018). *Entamoeba histolytica* infection in wild lemurs associated with proximity to humans. *Veterinary parasitology, 249*, 98-101.

Randremanana, R., Randrianirina, F., Gousseff, M., Dubois, N., Razafindratsimandresy, R., Hariniana, E. R., ...Richard, V. (2012). Case-control study of the etiology of infant diarrheal disease in 14 districts in Madagascar. *Plos one, 7*(9), e44533.

Randrianirina, F., Ratsima, E. H., Ramparany, L., Randremanana, R., Rakotonirina, H. C., Andriamanantena, T., …Talarmin, A. (2014). Antimicrobial resistance of bacterial enteropathogens isolated from stools in Madagascar. *BMC infectious diseases, 14*(1), 104.

Rasambainarivo, F. T., Gillespie, T.R., Wright, P.C., Arsenault, J., Villeneuve, A. and Lair, S. (2013). Survey of *Giardia* and *Cryptosporidium* in lemurs from the Ranomafana National Park, Madagascar. *Journal of Wildlife Diseases, 49*(3), 741-743.

Ravdin, J. I. (1989). *Entamoeba histolytica:* from adherence to enteropathy. Chicago, IL: The University of Chicago Press.

Ryan, U., & Cacciò, S. M. (2013). Zoonotic potential of Giardia. *International journal for parasitology, 43*(12), 943-956.

Salzer, J.S., Rwego, I.B., Goldberg, T.L., Kuhlenschmidt, M.S. & Gillespie, T.R. (2007). *Giardia* sp. and *Cryptosporidium* sp. Infections in Primates in Fragmented and Undisturbed Forest in Western Uganda. *The Journal of Parasitology, 93*(2), 439-440.

Schatz, G. (2000). Endemism in the Malagasy tree flora. *Diversity and endemism in Madagascar, 1*(9), 1-8.

Schwitzer, C., Mittermeier, R., Davies, N., Johnson, S., Ratsimbazafy, J., Razafindramanana, J., ...Rajaobelina, S. (2013). Lemurs of Madagascar: A strategy for their conservation 2013–2016. *Bristol, UK: IUCN SSC Primate Specialist Group, Bristol Conservation and Science Foundation, and Conservation International, 185*.

Scully, E.J., Basnet, S., Wrangham, R.W., Muller, M.N., Otali, E., Hyeroba, D., …Goldberg, T.L. (2018). Lethal respiratory disease associated in human rhinovirus C in wild chimpanzees, Uganda, 2013. *Emerging Infectious Diseases, 24*(2), 267-274.

Smith, J. M., & Meerovitch, E. (1985). Primates as a source of *Entamoeba histolytica,* their zymodeme status and zoonotic potential. *The Journal of Parasitology*, *71*(6), 751-756.

Tattersall, I. (2006). Origin of the Malagasy Strepsirhine Primates. *Lemurs: ecology and adaptation. Springer, New York*, 3-18.

Thompson, R. A. (2004). The zoonotic significance and molecular epidemiology of Giardia and giardiasis. *Veterinary parasitology*, *126*(1-2), 15-35.

UNICEF Madagascar. Madagascar WASH Investment Case: Executive Summary. Antananarivo, Madagascar.

WHO. World Health Organization (1997). Weekly Epidemiological Record: Amebiasis. Geneva, Switzerland.

WHO. World Health Organization (2018). International Travel and Health: Amoebiasis. Geneva, Switzerland.

Wolfe, N. D., Escalante, A. A., Karesh, W. B., Kilbourn, A., Spielman, A., & Lal, A. A. (1998). Wild primate populations in emerging infectious disease research: the missing link? *Emerging infectious diseases, 4*(2), 149.

Zhang, S., Kingsley, R. A., Santos, R. L., Andrews-Polymenis, H., Raffatellu, M., Figueiredo, J., …Bäumler, A.J. (2003). Molecular pathogenesis of *Salmonella enterica* serotype Typhimurium-induced diarrhea. *Infection and immunity, 71*(1), 1-12.

Zohdy, S., Grossman, M. K., Fried, I., Rasambainarivo, F., Wright, P. C., & Gillespie, T. R. (2015). Diversity and prevalence of diarrhea-associated viruses in the lemur community and associated human population of Ranomafana National Park, Madagascar. *International Journal of Primatology, 36*(1), 143-153.

TABLES AND FIGURES

Table 1: Previous research of enteric pathogens in humans and lemurs in and around Ranomafana National Park, Madagascar, completed in the Gillespie lab. These pathogens showed potential for zoonotic transmission as they were found in both humans and lemurs. This is not a comprehensive background of the research; *Giardia* and *Entamoeba histolytica* have been recorded in other studies outside of the Gillespie lab. Although *Giardia* was not found in lemurs, a number of limitations in the 2013 study could not rule out the potential for it in this study.

Pathogen	Prevalence (Humans)	Prevalence (Lemurs)
Shigella spp.	26.0-64.0% (Bublitz et al., 2015); 22.0% (Giordano et al., 2018)	4.0% (Bublitz et al., 2015)
Salmonella enterica	16.0-32.0% (Bublitz et al., 2015); 5.4% (Giordano et al., 2018)	8.0% (Bublitz et al., 2015)
Giardia sp.	N/A	0.0% (Rasambainarivo et al., 2013)
Entamoeba histolytica	N/A	4.0% (Ragazzo et al., 2018)

Table 2: Description of habitats and lemurs sampled in and around Ranomafana National Park, Madagascar. N represents number of *Microcebus rufus* trapped and sampled at each site. Forest and campsite lemurs were trapped in 2011 by an interdisciplinary team led by Dr. Gillespie including Deanna Bublitz, Fidy Rasambainarivo and Emo Redwood. Sarah Zohdy joined the lab in 2012 and oversaw the analysis of the samples.

Site	*Type*	*Sample size*
Valohoaka-Menarano (2011)	No regular overlap with humans	N = 9
Villages (2017)	Frequent overlap with local villagers	N = 21
Campsite/road (2011)	Frequent overlap with tourists and villagers	N = 21
		Total N = 51

Table 3: Samples from *Microcebus rufus,* brown mouse lemur, in Ranomafana National Park, Madagascar were tested for *Entamoeba histolytica, Salmonella enterica, Shigella flexneri,* and *Giardia lamblia* using pathogen PCR and gel electrophoresis protocols. Optimized lab protocols were used to determine annealing times and temperatures.

Pathogen	Gene	Target	Primer Sequence (5'-3')	Product Size
Entamoeba histolytica	SS rRNA (GenBank accession no. X64142)	Forward	GAA GCA TTG TTT CTA GAT CTG A	301 bp
		Reverse	CTC GTT CGT TAC CGG AAT TAA CC	
Salmonella enterica	invA	Forward	TAT CGC CAC GTT CGG GCA A	275 bp
		Reverse	TCG CAC CGT CAA AGG AAC C	
Shigella flexneri	ipaH	Forward	CTT GAC CGC CTT TCC GAT AC	610 bp
		Reverse	CAG CCA CCC TCT GAG AGT A	
Giardia lamblia	tpi	Forward (initial)	AAT AAA TIA TGC CTG CTC GTC G	530 bp
		Reverse (initial)	ATG GAC ITC CTC TGC CTG CTC	
		Forward (final)	CCC TTC ATC GGI GGT AAC TTC AA	
		Reverse (final)	GTG GCC ACC ACI CCC GTG CC	
	gdh	Forward (initial)	GAG GTC ATG CGC TTC TGC CA	599 bp
		Reverse (initial)	CGT CCA CTG GAG CCT CAC GGA	
		Forward (final)	ATG ACC GAG CTC CAG AGG CAC GT	
		Reverse (final)	CCC TCG GCC ACG AAC TTG AG	
	bg	Forward (initial)	AAG CCC GAC GAC CTC ACC CGC AGT GC	511 bp
		Reverse (initial)	GAG GCC GCC CTG GAT CTT CGA GAC GAC	
		Forward (final)	GAA CGA ACG AGA TCG AGG TCC G	
		Reverse (final)	CTC GAC GAG CTT CGT GTT	

Table 4: Pathogen-specific and general prevalence across forest, village, and campsite habitats in *Microcebus rufus,* brown mouse lemur, in Ranomafana National Park, Madagascar. N refers to total sample size at each site. Pathogen prevalence is expressed as a percentage, followed by (N positive/ total N at that habitat). Prevalence for *Giardia* is the same as for total disease because every sample with any pathogen tested positive for *Giardia*.

Site	**Anthropogenic influence**	**Number of samples**	**Positive for *S. enterica***	**Positive for *E. histolytica***	**Positive for *G. lamblia***	**Positive for any pathogen**
Forest	Very low	9	0	0	11.1% (1/9)	11.1% (1/9)
Village		21	4.8% (1/21)	0	47.6% (10/21)	47.6% (10/21)
Ambinandranfotoka		3	0	0	33.3% (1/3)	33.3% (1/3)
Mangevo	High contact with villagers	5	0	0	0	0
Miaranony		9	11.1% (1/9)	0	55.6% (5/9)	55.6% (5/9)
Bevoahazo		3	0	0	100% (3/3)	100% (3/3)
Beremby		1	0	0	100% (1/1)	100% (1/1)
Campsite	High contact with tourism and villagers	21	0	14.3% (3/21)	81.0% (17/21)	81.0% (17/21)
Total		51	2.0% (1/51)	5.9% (3/51)	54.9% (28/51)	**54.9% (28/51)**

Table 5: Comparison of infection prevalence in *Microcebus rufus* between sites near Ranomafana National Park, Madagascar. Forest-village and forest-campsites statistics were produced using the general logistic regression model with forest as the reference group; the village-campsite statistics were produced with the multiple comparison test with forest as the reference group. Because we expect directionality within the site variable (infection in campsite > village > forest), we included the one-tailed test directional P-value.

	Forest – village	**Forest – campsite**	**Village - campsite**
Estimate	1.984	3.526	1.542
Standard error	1.147	1.197	0.707
Z-value	1.730	2.945	2.182
Two-tailed test P-value ($\alpha = 0.05$)	0.084.	0.003**	0.029*
One-tailed directional P-value ($\alpha = 0.05$)	0.042*	0.002**	0.015*

Table 6: Assessment of effect of variables on infection *in Microcebus rufus* near Ranomafana National Park, Madagascar. This model was produced using a general logistic regression model. As the P-values are not significant, these variables appear to not have a large effect on outcome of infection. Sex was a dichotomous variable (Female = 1, Male = 0); condition was (weight/length from nose to base of tail), as we expected this to be a good estimate of health.

	Sex (Female)	**Condition (Weight/length)**
Estimate	0.278	-1.268
Standard error	1.099	1.128
Z-value	0.253	-1.124
Two-tailed test P-value ($\alpha = 0.05$)	0.800	0.261

Figure 1: Locations where brown mouse lemurs (*Microcebus rufus*) were trapped and sampled in 2011 (green and red) and 2017 (yellow) in and around Ranomafana National Park in Southeastern Madagascar.

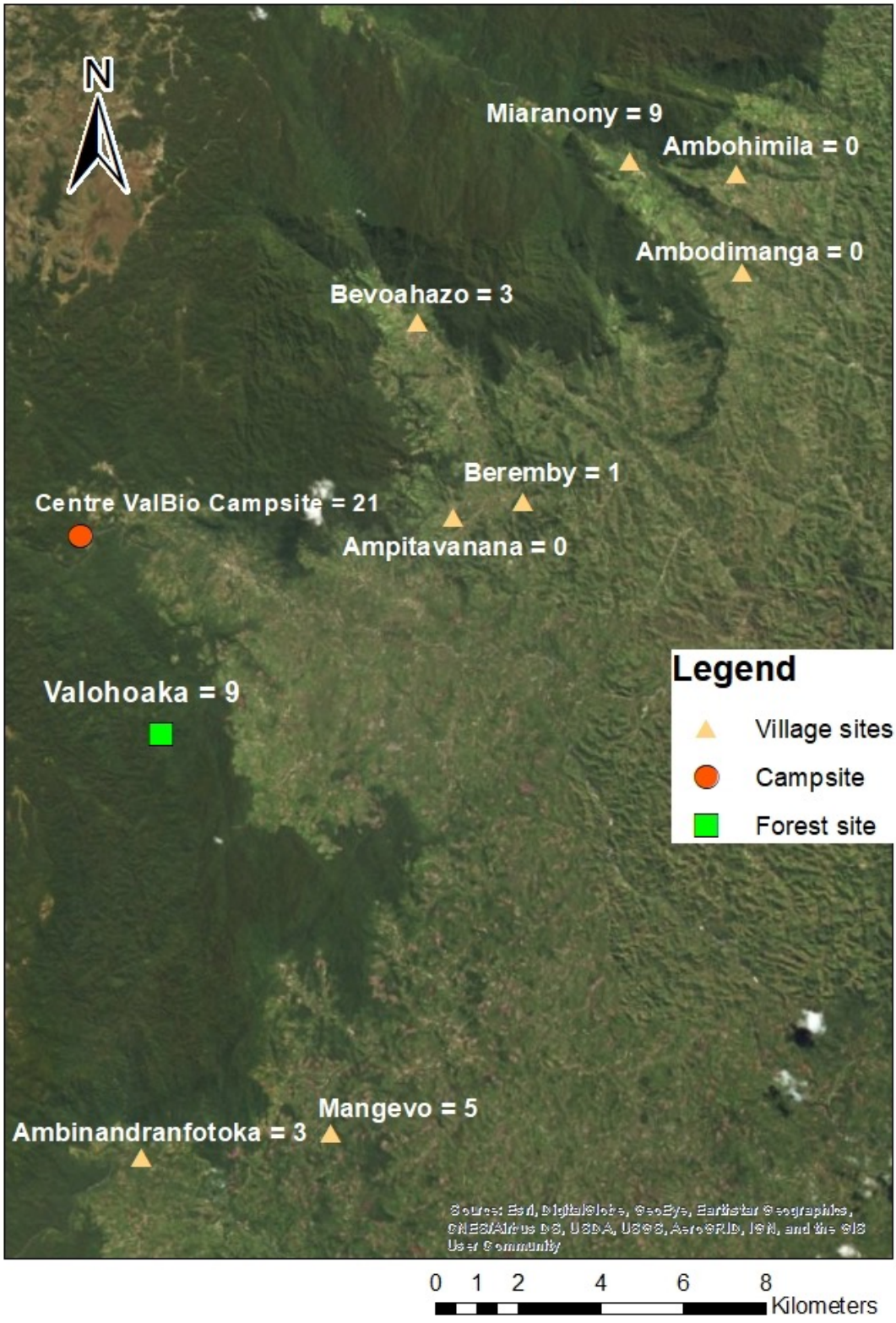

Figure 2: A typical trapping setup for *Microcebus rufus*, brown mouse lemur, on a branch at a village site outside of Ranomafana National Park, Madagascar.

Figure 3: A close-up of the study specimen, *Microcebus rufus* after being trapped and sampled at a village site outside of Ranomafana National Park, Madagascar.

Figure 4: Bar chart of infection prevalence in *Microcebus rufus* by site in Ranomafana National Park, Madagascar.

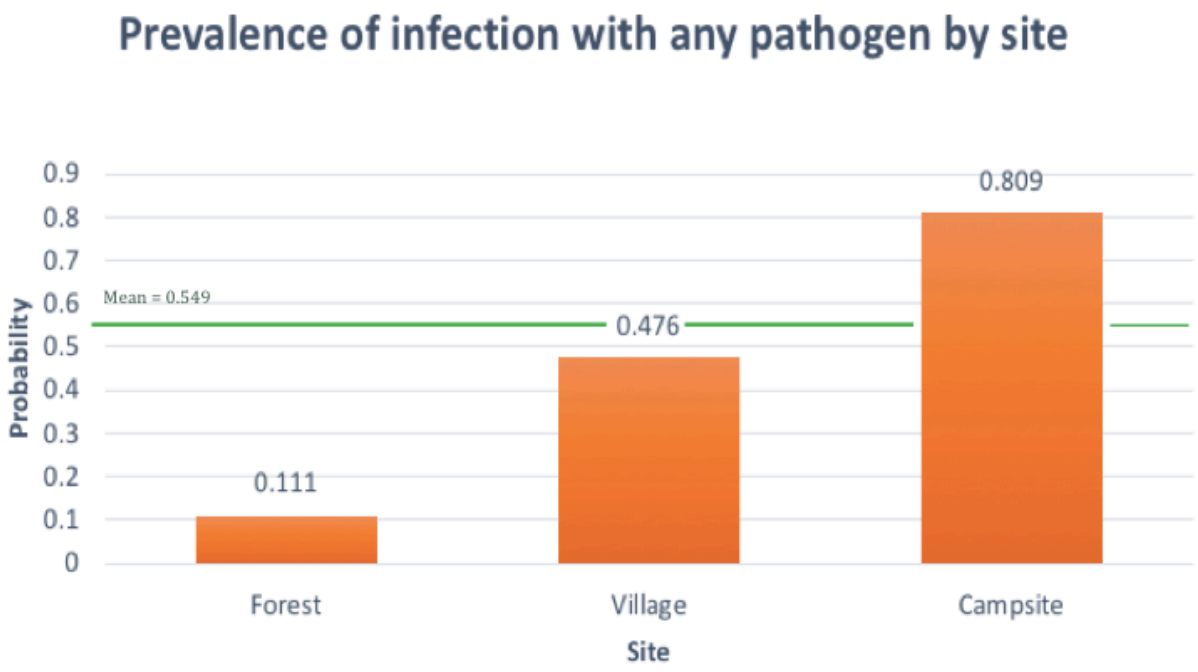

Figure 5: Map of overall infection prevalence by village near Ranomafana National Park, Madagascar.