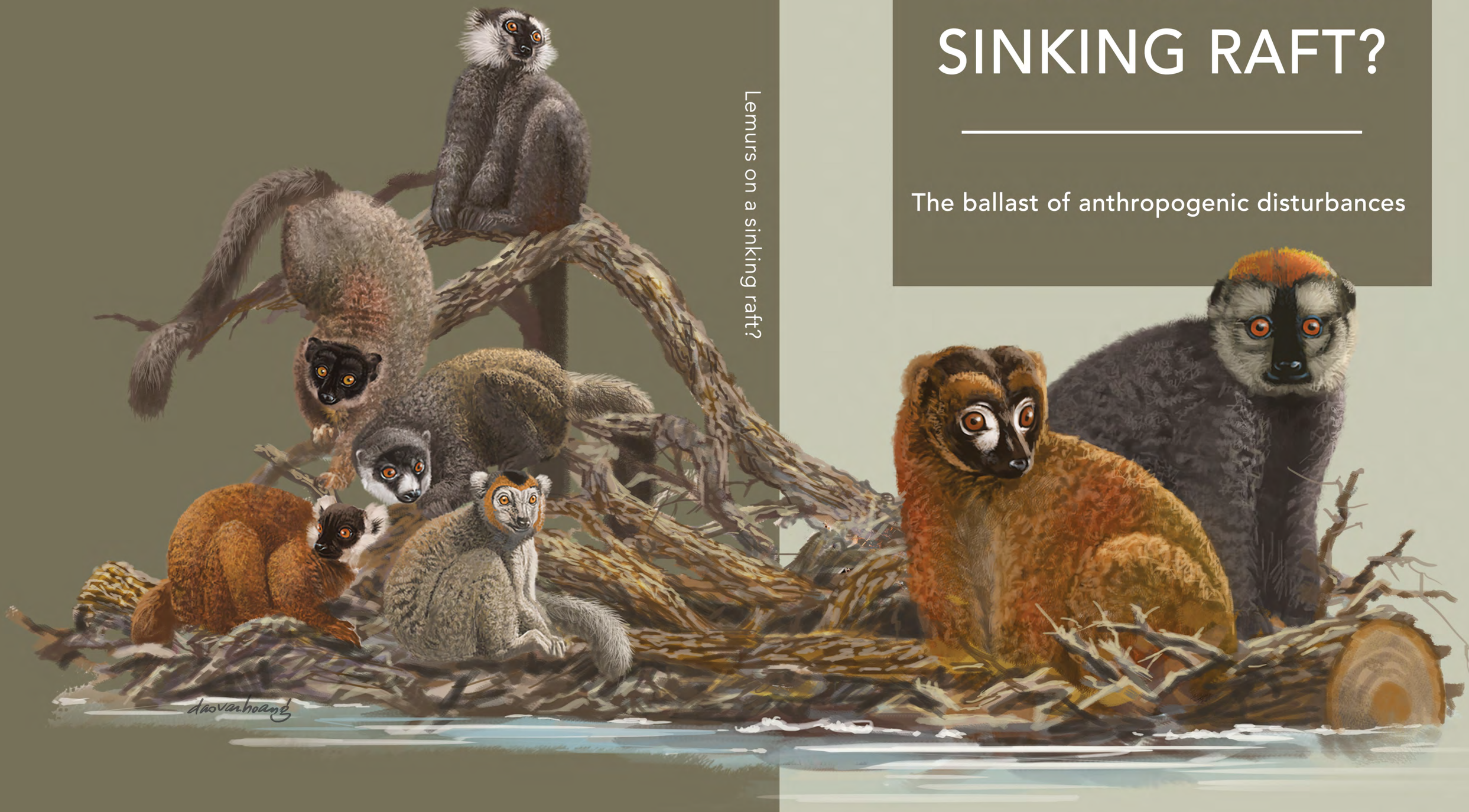
Iris I. de Winter

LEMURS ON A SINKING RAFT?

The ballast of anthropogenic disturbances



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Thesis committee

Promotor
Prof. Dr H.H.T. Prins
Professor of Resource Ecology
Wageningen University & Research

Co-promotors
Dr I.M.A. Heitkönig
Assistant professor, Resource Ecology Group
Wageningen University & Research

Dr W.F. van Hooft
Assistant professor, Resource Ecology Group
Wageningen University & Research

Other members
Prof. Dr M. Naguib, Wageningen University & Research
Prof. Dr E.H.M. Sterck, Utrecht University
Dr N. Tagg Nama, Conservation Royal Zoological Society of Antwerp, Belgium
Dr E. Meijaard, The University of Queensland, Australia

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Thesis

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Iris I. de Winter
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PREFACE

Wild primates are fascinating creatures that occupy a special place in the hearts of people around the globe. The clade of lemurs evolved in isolation, exclusively on the island of Madagascar, yet the magnitude of its behavioural and morphological diversity rivals that of the monkeys and apes found elsewhere in the world. One truly feels fascinated when near these agile tree dwellers as they serenely warm up in the sun, groom each other, or move through the lush vegetation, observing us observing them with a mirrored curiosity vividly apparent in their striking eyes. It is worrisome to imagine a world without these charming animals; but, with a combination of ongoing research, clear communication, and the development and implementation of effective conservation measures, we may not have to.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Like all of the earth's natural ecosystems, tropical forests are influenced by a wide range of anthropogenic and natural impacts (Barlow et al., 2016). Anthropogenic disturbances to the natural environment date back to early human occupation in the tropics. Especially when humans started using fire, about 1.8 million years ago (mya), landscape modification and biotic adjustments became more intense (Vié et al., 2009). The shift from hunting-gathering to farming was one of the most important factors that has sparked land conversion and modification (Goudie, 2013). Given the tendency of humans to directly and often considerably alter and impact the environment, nearly all currently-existing forests, including tropical forests, have experienced anthropogenic forest exploitation (Bicknell et al., 2015). Although tropical forests form less than 10 percent of the world's land area, half of all animal and plant species and even 90 percent of all nonhuman primates that are known today rely on these forests for their subsistence (Mayaux et al., 2005; Whitmore et al., 1992). Because of ongoing loss of tropical forests, all these species have become vulnerable, and population densities and distributions of many species are in decline (Chapman et al., 2007; Cowlishaw and Dunbar, 2000; Fashing, 2002).

Human interferences with the environment are not limited to mainland forests. Human discovery of and arrival on numerous remote islands within the world's oceans has drastically changed many island ecosystems. Due to their isolation, unique geology, and climate regimes, islands are often home to a diverse array of ecosystems that host rare and endemic flora and fauna (Yoder and Nowak, 2006).

In addition to anthropogenic impacts, local natural disturbances of tropical forests have occurred over large geographical time scales. These disturbances range from small-scale events, such as tree-falls, to larger-scale processes, such as erosion, fire, and annual flooding. Tropical forests have always been highly impacted by other natural phenomena: geological events (e.g., earthquakes, landslides, and volcanic eruptions), atmospheric events (e.g., tropical cyclones, droughts, and lightning), climatic variations (e.g., ice ages and sea level fluctuations), and other hazards (e.g., disease epidemics and insect infestations). In addition, environmental circumstances, like soil and climate conditions as well as topography, influence the makeup and function of forest ecosystems (reviewed in Chazdon 2003). Anthropogenic disturbances and natural challenges interact with each-other in complex ways and together shape the ecosystems in which many unique plant and animal species reside.

This chapter forms a general introduction that sets the scene by presenting the

theoretical framework of this thesis, including an overview of the available literature. I outline the scope; introduce the aim, main questions, and hypothesis; and provide the study design, including a biological background of the study species and information on the geographic locations.

THE ISLAND OF MADAGASCAR

Despite its proximity to Africa, the island of Madagascar has a very different biotic and human history than that of the mainland. Continental drift led to its isolation and, together with India and Antarctica, Madagascar split from the Africa-South America landmass approximately 165 mya, reaching its current position around 120 mya (Coffin and Rabinowitz, 1992). The estimated continental breakup of Madagascar and India was 88 mya (Storey et al., 1995). Over millions of years, a number of distinct biomes have developed on the island.

On a geologic timescale, transportation via rafts of floating debris has played an important role in dispersal of organisms around the globe, especially for non-swimming and non-flying organisms (Heatwole and Richard, 1972). For example, New World Monkeys originated in Africa and rafted during the Eocene to South America (Bond et al., 2015). Tropical storms and flooding events are known to rip away floating lumps of earth, complete with living vegetation and micro-ecosystems (Simpson, 1940). Vertebrate colonisation of Madagascar most likely occurred via this type of dispersal, on floating rafts of vegetation across the ocean (Ali and Huber, 2010). This theory also holds for lemurs, a clade of endemic Strepsirrhine primates that arrived on the island between 60 and 50 mya (Yoder and Yang, 2004, Box 1.1). During this geological period, both Madagascar and Africa were located approximately 1650 kilometres south of their present positions and ocean currents ran in eastern direction from Africa towards Madagascar (Ali and Huber, 2010). Natural rafts for a lemur ancestor could therefore have been formed from vegetation lining rivers on the east coast of Africa -for example, from riverbanks of the Zambezi or from the shoreline of northeastern Mozambique and Tanzania- and transport should have been towards the northwest coast of Madagascar (Stankiewicz et al., 2006). By floating on such rafts of vegetation, a lemur ancestor, possibly a single pregnant female, migrated from mainland Africa, transported by ocean currents across the approximately 400 km wide Mozambique Channel, and stranded on the enormous 'raft' called Madagascar (Ali and Huber, 2010).

About 40 mya, the currents gradually changed southwards, thereby putting an end to any further oceanic transport and isolating Madagascar and its inhabitants from other landmasses (Ali and Huber, 2010). Lemurs evolved in isolation, independent of competition from monkeys, apes, or other ecologically-competitive large-bodied mammals (Yoder and Nowak, 2006). Madagascar varies widely in climate, seasonality,

and geology across the island (Fig. 1.1, Yoder & Nowak 2006); to avoid competition with conspecifics, lemur populations were forced to limit niche overlap as much as possible, driving them into Madagascar's numerous environmental niches. This led to the adaptive radiation of more than a hundred different species (Yoder and Yang, 2004), representing 29 percent of all primate families and 20 percent of the genera and species that are known today (Table 1.1, Bowman et al. 2018). Lemurs are one the most diverse and geographically isolated groups of primates in the world. They are only found on Madagascar, and are recognised as keystone species in some of the most endemic and threatened ecosystems in the world (Schwitzer, 2014).

Box 1.1 The lemur clade. Lemurs - *Kingdom: Animalia, Phylum: Chordata, Class: Mammalia, Order: Primates, Suborder: Strepsirrhini, Family: Lemuridae*

Lemurs originated on mainland Africa and split from a shared, ancestral primate 62 to 65 mya (Yoder and Yang, 2004, Fig. 1.2). They belong to the suborder Strepsirrhini that consist of three infraorders: Lemuriformes (lemurs), Chiromyiformes (aye-aye), and Lorsiformes (lorises and galagos). When compared to primates in the suborder Haplorrhini, lemurs, and all other Strepsirrhini, exhibit a number of traits that are considered ancestral ('primitive') for the order Primates. Those traits mostly relate to skull morphology, brain arteries, and the jaw and dental structure of animals. For example, all lemurs have a toothcomb in their lower jaws, which is composed of their lower incisors and (sometimes) canines and is used for grooming and feeding (Fleagle, 2013). For grooming, they also have a grooming claw on the second toe of each foot. Lemurs seem to rely more on olfaction than on vision for finding food and for communication with conspecifics. For example, they have a moist rhinarium (i.e., the area around the nose) and curved bones in the nasal cavity, both of which enhance their sense of smell (Fleagle, 2013). Furthermore, the lack of a *fovea centralis* in the retina, which is needed for sharp vision, suggests a reduced visual acuity when compared to Haplorrhines. Most lemurs also have a *tapetum lucidum*, a reflective layer in the back of the eye that aids in seeing at low light levels, but also reduces visual acuity (Martin, 1990).

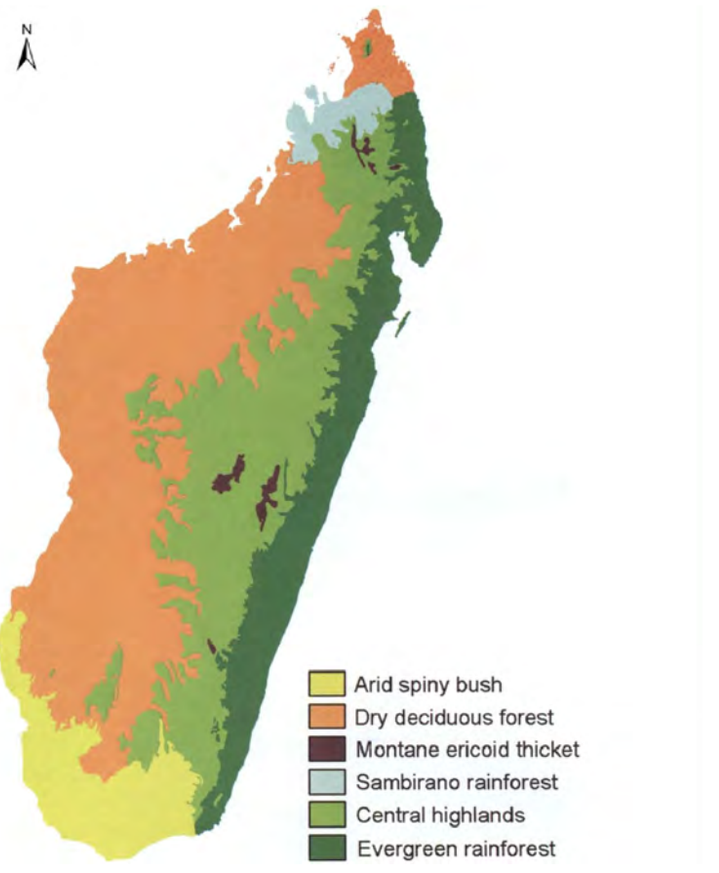


Figure 1.1: Illustration of the distributions of the different biomes in Madagascar (*retrieved from Yoder & Nowak 2006*).

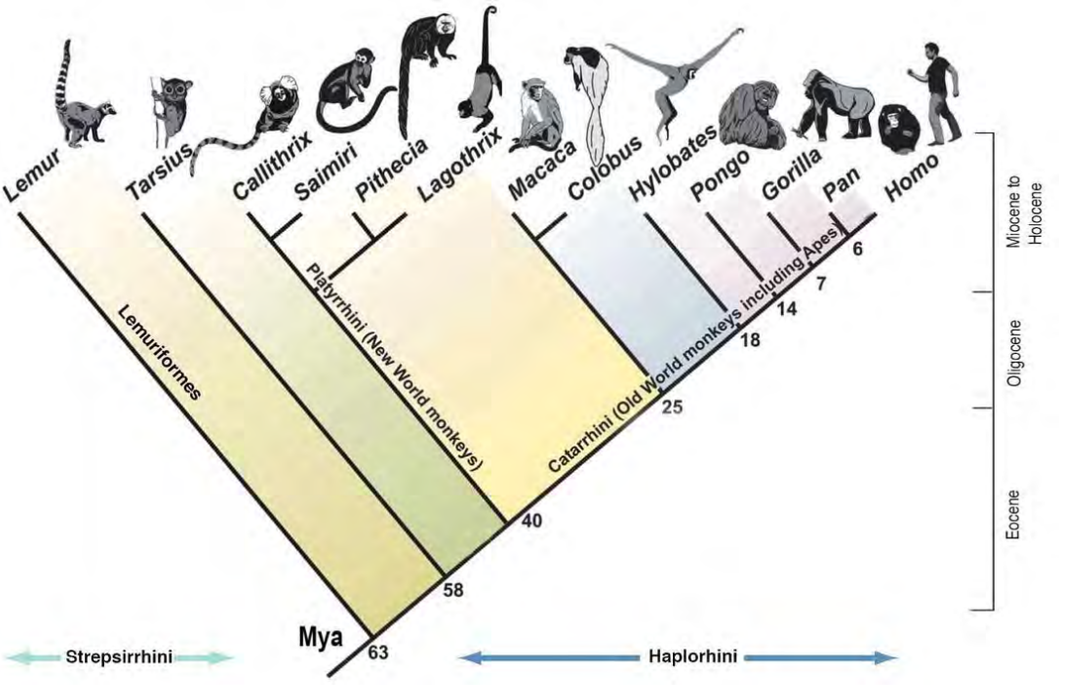


Figure 1.2: Phylogenetic tree showing the lineage of living primates. *Retrieved from http://anthropologyiselemental.ua.edu/, 11 Feb. 2018.*

Table 1.1: Global primate diversity. Total number of families, genera, and species and the percentage of threatened species worldwide. Adapted from *http://www.primate-sg.org, last updated: 30 Sept. 2014, retrieved: 26 Jan 2018.*

Region	Families	Genera	Species	Threatened (%)
Africa	4	25	111	31.5%
Asia	5	19	119	63.8%
Madagascar	5	15	101	88.5%
Neotropics	5	19	165	37.4%
Total	**17**	**76**	**496**	**50.4%**

ANTHROPOGENIC AND NATURAL DISTURBANCES

While dispersal via long-distance rafting has occurred throughout the history of life on Earth, it has always been a rare phenomenon (Simpson, 1940). However, humans started 'rafting' as well, and the first human settlers arrived on Madagascar via canoes from the Sunda islands between 200 BC and 500 AD, which was relatively late when compared to colonisation events elsewhere in the world (Burney, 1997). A variety of ethnic groups colonised the island and since their arrival, major anthropogenic landscape modifications and ecological changes have occurred. Humans modified the landscape with fire, forest clearance, pastoralism and cultivation, and with the introduction of exotic plant and animal species, which caused major consequences for the indigenous species on the island. Between 1950 and 2000, forest cover in Madagascar further decreased by 40 percent (Allnutt et al., 2008). Due to growing human populations, deforestation currently proceeds at rates of 1 to 2 percent per year (e.g., Harper et al., 2007; Scolozzi and Geneletti, 2012; Zinner et al., 2014) and nowadays, only about 10 to 20 percent of the original forest cover remains (Dufils, 2003; Goodman and Benstead, 2005).

Systemic issues, such as poverty and repeated political instability, have played major roles in the extensive habitat loss in Madagascar (Keane et al., 2011; Ormsby and Mannle, 2006; Pawliczek and Mehta, 2008; Zinner et al., 2014). People on the island are exceptionally poor in terms of housing, health care, security, energy, education, employment opportunities, and income. Most people live on less than one to two USD a day. With a GDP per capita of 415.8 USD (WTTC, 2017), they need to extract essential resources from the natural environment for their subsistence, i.e., food for themselves and their livestock, water, fuel, and housing materials (Harvey et al., 2014). Madagascar has undergone large-scale and rapid habitat loss, mainly due to traditional and unsustainable farming methods. The most practiced agricultural system by both locals and corporates is slash-and-burn (*tavy*) for rice cultivation (Scales, 2014; Stifel et al., 2003; Styger et al., 2007). Also, the practice of cattle (Zebu) grazing is an important contributor to land conversion from forest to grassland, through fire, and to further soil

degradation throughout Madagascar.

Like in other tropical rainforests, Madagascan soils are low in nutrients, and the usability of deforested agricultural land does not last long. The soils in deforested sites in Madagascar generally contain little organic matter and are low in nutrients, have a high pH, and differ from other tropical soils in their relatively low levels of many minerals and soluble salts (Stoop et al., 2002). After only few rotation cycles, the poor soils can no longer support crops and only some grasses for cattle ranching or barren soil remains (Styger et al., 2007). Non-sustainable commercial timber extraction for precious hardwood, such as rosewood (Barrett et al., 2010), is also an important cause of deforestation, and can disrupt a complete forest ecosystem (Ballet et al., 2009; Burivalova et al., 2015). Mining for metals and minerals, including sapphires and cobalt, and drilling for petroleum also threatens many forests habitats (Yager, 2004). Furthermore, charcoal production leads to both deforestation and the replacement of forest by eucalyptus plantations (Minten et al., 2013). Forest clearing and logging causes canopy disruptions and exposes top soils, which leads to further problems, including heavy soil erosion, nutrient leaching, flooding of rivers, and dry top soils that are depleted of most of their nutrients (Buckman and Brady, 1960; Durbin and Ratrimoarisaona, 1996).

All these habitat modifications can impair the resilience of forest systems (Laurance, 2015). Resilience is the capacity of an ecological forest system to alleviate external disturbances and to retain essential structures, processes, and functionality, despite the forced changes (Walker et al., 2004). A lower resilience, therefore, increases the vulnerability of forests when further exposed to natural or anthropogenic disturbances or to other forms of stress (Turner et al., 2003). Specifically, such historic and actual natural environmental alterations on Madagascar include climatic changes, fluctuating ocean levels, volcanic eruptions, seasonal fires, and extreme weather phenomena.

Anthropogenic habitat modification reduces the effective space available for species to exist (Irwin et al., 2010) and is the main cause of biodiversity loss in Madagascar (Scales, 2014; Stifel et al., 2003; Styger et al., 2007). One group of animals that is particularly impacted by these modifications, and is therefore highly threatened, is lemurs, with 75 percent of the species falling into the Critically Endangered and Endangered categories (IUCN, 2016). Since the arrival of humans, 17 lemur species in eight genera have gone extinct due to human impacts, like hunting and habitat destruction (Burney et al., 2004; Mittermeier et al., 2006). Furthermore, although lemur hunting is often taboo (*fady*) in the Malagasy culture, hunting lemurs for bush meat still occurs due to the lack of alternative protein sources and the emergence of the commercial bush meat and pet trade (Randrianandrianina et al., 2010). The future of Madagascar's biodiversity is uncertain and lemurs are currently

recognised as the most threatened group of large vertebrates in the world (Mittermeier et al. 2006, Schwitzer 2014, Table 1.1). Due to anthropogenic habitat change, most lemur populations nowadays live in 'islands' of forests, surrounded by an 'ocean' of humanity, with settlements and agricultural land right up to the islands' boundaries (Rowe and Myers, 2016). These anthropogenic threats within this islands-of-forests setting, as well as many external natural pressures, such as yearly cyclones and prolonged droughts, challenge the stability of the presumably safe 'raft' that lemurs have used as a refuge for millions of years. Furthermore, lemur habitats show strong differences across Madagascar, which is the result of an interaction between a mountain range that runs in north-south direction and an east-west and north-south rainfall gradient (Irwin et al., 2005). In the associated different vegetation zones, lemurs face strong variation in climatic conditions, seasonality, and geology, leading to large variations in food availability that, in combination with anthropogenic disturbances, further challenge their health and survival (Irwin et al., 2010).

AIM AND RELEVANCE

Globally, deforestation and degradation of natural forests continue to threaten the persistence of biodiversity (Gibson et al., 2011; Vieilledent et al., 2013). In order to overcome the many threats species are facing, successful management of nature has never been more important. Studies on wild primates are of particular interest, as the primate taxon has a high conservation status and ongoing habitat modifications form a serious threat to many primate species (Table 1.1). Disturbances may exert stress and can affect the overall condition, health status, and immune functioning of wildlife species, including lemurs (Chapman et al., 2005). This makes species less likely to withstand additional pressures, thereby threatening their survival and reproduction.

Most studies on wild primates have focused on resource competition and predation as driving forces of primate ecology, fitness, and social behaviour. While multiple studies have acknowledged that external disturbances play an eminent role in primate ecology, the general theoretical literature on this subject, and specifically in the context of Madagascar, is inconclusive on how all these potential threats impact lemurs (Gardner et al., 2007). Although lemurs have been extensively studied in the past few decades, an integrated perspective of actual health effects of anthropogenic and natural challenges on multiple lemur species is still lacking. Especially the following health parameters: microbiota composition, parasite infections, and immunocompetence, have remained relatively unexplored within these species and is debated within the scientific community. Given that lemur survival is currently threatened by intense anthropogenic pressure and additional natural impacts, examining the effects of such disturbances on the abundances and health of these endemic primates is an urgent issue (Harper et al., 2007; Scolozzi and Geneletti, 2012;

Wright, 1999).

In this thesis, I aim to provide an overview of the complex relationships between multiple anthropogenic and natural impacts on the one hand and the presence, behaviour, and health of lemurs across the island of Madagascar on the other. My principal question is: how do multiple anthropogenic disturbances impact forest structure, lemur presence and behaviour, and the health of lemurs? The overriding hypotheses of this thesis are (1) that anthropogenic disturbances (i.e., selective logging) alter lemur encounter rates and facilitate the coexistence of closely related species, and that (2) these disturbances as well as natural challenges influence the lemurs' microbiota composition, gastrointestinal (GI) parasite levels, and MHC II *DRB* diversity. The specific questions in each chapter are provided as well (Box 1.2), and the specific hypotheses can be found in the associated chapters. In the synthesis, I discuss other challenges that biodiversity conservation in Madagascar is facing in the near future, and propose potential solutions to ensure the long-term survival of lemurs.

Results of this study can help to advance health, molecular, and biological science of primates, and provide a good basis for further investigations that aim to unravel the impact of natural or human-induced habitat alterations on primate populations. To my knowledge, this study will be the first in its integrated perspective, linking the impacts of anthropogenic and natural challenges to the occurrence and multiple health aspects of lemurs on different geographic scales. This thesis addresses major questions in different disciplines within applied ecology, including community, behavioural, and conservation ecology. This work is of relevance for science, as it contributes to our understanding of lemur responses to several forms of ecological stress, including anthropogenic disturbances. The value of this study is further enhanced by its relevance for and potential contribution to the management and conservation of wild lemur populations in Malagasy forests.

Box 1.2 Overview of the main questions of this thesis per chapter. A more complete description of these questions can be found in the associated chapters. I provide an overview of the answers to these questions in chapter 7, Box 7.1.

Chapter 2
Can the impact of past logging still be discerned in forest structural characteristics?
Have previously logged forests recovered into functional lemur habitat?

Chapter 3
How can the coexistence of the congeneric lemur species *Eulemur rufifrons* and *E. rubriventer* be explained?

Chapter 4
To what extent does location, species, sex, and age influence the faecal bacterial microbiota composition in lemurs?

Chapter 5
How do geographic location, seasonality, and anthropogenic disturbances influence parasite infections and faecal bacterial microbiota composition in lemurs?
Is there an interactive effect between GI parasites and microbiota?

Chapter 6
What is the variability of immunologically important genes (i.e., MHC class II *DRB*) among geographically separated *Eulemur* species?

FOREST DISTURBANCE AND LEMUR PRESENCE

ANTHROPOGENIC DISTURBANCES

Throughout the tropics, development activities, logging, and agricultural expansion have resulted in the conversion of previously continuous forests into landscape mosaics of forest fragments, secondary vegetation, and agricultural areas (Gardner et al., 2007). These human-induced changes limit the potential for forest regeneration, can disrupt forest stability, and can have long-lasting impacts on forest structure and animal abundances, including lemurs (Aber et al., 2002; Laurance, 2015; Malmer and Grip, 1990). The relations between logging, forest structure, and lemur abundances are complex and reflect varying causalities (Lehman et al., 2006). Studies examining the impact of selective logging on other primate communities did not find consistent trends in primate abundances (Johns, 1992). Also, studies on lemur diversity within Madagascar's rainforests have yielded conflicting results (Ganzhorn, 1988, 1992, 1995).

In selectively logged forests, large mature trees of commercial value are usually removed (McElhinny et al., 2005). For that reason, past logging can result in a lower average tree diameter, tree height and crown volume, and higher tree densities due to the increased emergence of tree saplings (Gibson et al., 2011). Logging can reduce forest quality and food availability for primate communities (Chapman et al., 2000; Struhsaker, 1997; White et al., 1995). Larger primate species with specialised diets and slow reproduction are typically most vulnerable to such forest disturbances, when compared to more generalist species (Cowlishaw et al., 2009; Newbold et al., 2014).

In contrast, some small-scale disturbances can be beneficial for both folivorous and frugivorous species, as fruit production and leaf quality increase following increased sunlight exposure at the understory level (Ganzhorn, 1995). Numerous studies have found a positive relationship between increased heterogeneity in multiple forest characteristics following logging events and the diversity in potential habitats, essential food resources, and shelter for numerous animal communities (e.g., Dunn, 2004; Yang et al., 2015). Some primate species are also able to adapt to forest changes. For example, primates can alter their social group sizes when the quality of food patches change, often to reduce the costs of intragroup competition (Chapman and Chapman, 2000; Isbell, 1991). The literature suggests that the responses of primate communities to anthropogenic habitat alterations depend on the type and intensity of the disturbance, the time since modification took place, and the extent of the primates' ecological and behavioural flexibility in response to changes (Chapman et al., 2004; Johns and Skorupa, 1987; Marsh et al., 1987).

Although some lemur species seem to cope with human-induced forest alterations, habitat loss and fragmentation are among the most pervasive causes of declining lemur populations and biodiversity loss in Madagascar (e.g., Irwin et al. 2010, Laurance et al. 2000, Gardner et al. 2010). As species responses to disturbance can be very different across ecoregions (Irwin et al., 2010), it is important to have a local understanding of the responses of forests and lemurs to such human disturbances (Kinnaird, 1992; Wieczkowski, 2003). In **chapter 2**, I investigate how forests and lemurs respond to different levels of selective logging and, thereby, evaluate the flexibility of lemurs in a changing environment.

COEXISTENCE OF CONGENERS

The mechanisms for the coexistence of species within ecological communities remain one of the major topics in community ecology (Dammhahn and Goodman, 2014; Dammhahn and Kappeler, 2008; Rakotondravony and Radespiel, 2009). In primate species, the geographic coexistence of congeneric species is rare (Houle, 1997), but it is relatively common in lemurs (Kamilar et al., 2014). The causal mechanism behind the coexistence of closely related lemur species, and especially congeners (i.e.,

species that belong to the same genus), is a much debated subject and is not easily understood (Chase and Leibold, 2003; Dammhahn and Goodman, 2014). Due to their recent common ancestry, congeneric species are generally more similar to each other in their biology, ecology, and morphology when compared to more distantly related taxa (Sfenthourakis et al., 2005). As a result, many similarities within their fundamental niches may be present (Schoener, 1982; Sinclair et al., 2006), while species that share the same habitat should show greater differences in their niches in order to coexist. Interspecific competition should therefore constrain the coexistence of such species (Futuyma, 2013).

Several mechanisms are known to promote species coexistence. First, spatial heterogeneity of forests can result from natural disturbances (van der Maarel 1993) and anthropogenic disturbances, including selective logging (de Winter et al., 2018a; Questad and Foster, 2008). Such landscape variations, caused by disturbances, can result in a patchy distribution of species, thereby limiting competition and hence promoting coexistence in ecological communities (Roxburgh et al., 2004). Second, differentiation along the major niche dimensions, i.e., food resources, space, and time, is needed to lower competition between species (Amarasekare, 2003; Pianka, 1973; Sauther, 1993; Schoener, 1974). Third, interspecific competition can shape niche differences between closely related lemurs (Rakotondravony and Radespiel, 2009). In **chapter 3**, I test whether it is logging-initiated landscape heterogeneity, niche differentiation, and/or interaction patterns that limit interspecific competition among congeneric lemur species and, hence, facilitate their coexistence. To this end, I measured species encounter rates in five different sites within a rainforest in southeast Madagascar, which had experienced different levels of selective logging in the past.

LEMUR HEALTH

Lemurs face high levels of natural and anthropogenic impacts on their health and survival. Being forced into suboptimal habitats due to ongoing forest degradation and their potentially compromised nutritional status, stress levels are likely increased, which can affect the lemurs' immune status and can make them more susceptible to diseases (Chapman et al., 2005). The ecological constraints imposed by disturbances, in turn, can influence diet composition, reproduction, and mortality rates, and ultimately, the survival of lemurs (Menon and Poirier, 1996). In this thesis, I focus on three important health parameters to explore the potential consequences of both natural and anthropogenic impacts on lemur health: the faecal bacterial microbiota composition, parasite prevalence, and MHC II *DRB* diversity.

MICROBIOTA COMPOSITION

The diversity and composition of the intestinal microbial community of mammals contributes to the overall health of animals through modulation of their immune system, facilitation of food digestion, competition with pathogenic microorganisms, and production of metabolites (Clemente et al., 2012; Kabat et al., 2014; Patterson et al., 2014). Hence, identifying the factors and underlying processes that shape the intestinal microbiota is important. Studies on other primate species revealed that the microbiota composition can be highly variable, also intra-individually, and mostly depends on the available diet (Amato et al., 2015; Ren et al., 2015; Yildirim et al., 2010). Based on existing knowledge, however, it is not clear to what extent host species and environmental factors influence intestinal microbial composition under natural conditions. In **chapter 4**, I therefore investigate the faecal microbiota composition of multiple wild *Eulemur* species across different biogeographical regions.

HELMINTH-MICROBIOTA ASSOCIATIONS

All mammals can be infected with a wide variety of parasite species. These parasites are classified into micro- and macroparasites. Microparasites complete their life cycle within a host organism and usually cannot be seen with the naked eye (e.g., viruses and bacteria) (Anderson and May, 1981). In contrast, macroparasites generally spend a portion of their lifecycle detached from their primary host and can usually be seen with the naked eye (e.g., ticks, mites, nematodes, and flatworms). Gastrointestinal macroparasites can be present within a host's digestive tract and spread through the faecal-oral route, which involves ingestion of contaminated soil or food (Nunn et al., 2011). In this study, I focus on GI macroparasites: nematodes (Anderson and May, 1991; Samuel et al., 2001), as they can be isolated and identified from non-invasively collected faecal samples.

The distribution of parasite infections in wild host populations is influenced by a number of factors, including host susceptibility and exposure (Moore and Wilson, 2002). The nematodes that are the focus of this thesis spend part of their life cycle outside the host and are therefore exposed to environmental conditions that shape temporal variations in parasite infections. It has been shown that some nematodes have an accelerated development and increased reproduction and survival rates in wet and warm conditions (Benavides et al., 2012; Nunn and Altizer, 2006) and desiccate more frequently under dry circumstances (Huffman et al., 1997). Parasitism can impact the host's health, behaviour, and survival, thereby influencing evolutionary processes and population dynamics (Ramanan et al., 2016). In addition, parasites are known to affect the host's reproduction directly through pathologic effects and mate choice as well as indirectly by impairing nutrition and creating energy deficits (Leclaire and Faulkner, 2014). Although some parasite infections generally do not cause clear

clinical signs of disease (Burthe et al., 2008), parasites and the diseases they carry can have profound consequences for the health and fitness of host organisms. Although understanding the determinants of parasite infections in wild primates is relevant, the underlying mechanisms of the aggregated distributions of parasites within most host populations is still unclear (Chapman et al., 2005; MacIntosh et al., 2010). It is therefore essential to explore the current distributions of these parasites within lemur populations in a changing environment.

Environmental factors can influence both microbial composition and parasite prevalence (Barelli et al., 2015; Maurice et al., 2015). Climatic seasonality has been identified as an important driver of temporal variation in several wild primate species (Benavides et al., 2012; Huffman et al., 1997). However, studies investigating these links have yielded different outcomes (Aivelo et al., 2016; Amato et al., 2015; Barrett et al., 2013). Microbiota and parasites co-inhabit the GI tract and have evolved in close association, suggesting that they have the potential to influence each other (Kreisinger et al., 2015). Research on this interplay between host, parasites, and the microbiome has increased over the last decade (Mutapi, 2015) and recent studies in humans showed associations between nematode infections and changes in the GI microbiota structure (Kay et al., 2015; Lee et al., 2014; Morton et al., 2015). However, this observation is not consistent across studies (Cantacessi et al., 2014; Cooper et al., 2013) and requires further study in wild mammals. In **chapter 5**, I assess the effects of seasonality and forest disturbance on GI parasites and bacterial microbiota composition and address the interactive effects of parasite prevalence and the microbiome.

IMMUNOCOMPETENCE

Ongoing forest disturbances in Madagascar can increase stress levels in lemurs, thereby impacting the animals' immune functioning and susceptibility to diseases. An important class of immune genes for parasite recognition and resistance is the Major Histocompatibility Complex class II (MHC II). MHC II genes code for proteins that bind parasite-derived antigens and present them to lymphocytes (T-cells). Hereby, an adaptive immune response is initiated (Janeway et al., 2004). MHC II genes can be highly polymorphic (Parham and Ohta, 1996; Vogel et al., 1999) and animals with such a high variability are considered to recognise a wider variety of pathogen peptides compared to animals with a lower MHC II variability (Doherty and Zinkernagel, 1975). Thus, genetic variation in functionally important MHC gene families plays a central role in vertebrate immunity and in the viability and long-term survival of wildlife populations (Piertney and Oliver 2006; Radwan et al. 2010; Siddle et al. 2007). Especially for small and isolated populations, quantifying and monitoring *DRB* diversity in lemur species is therefore important to evaluate lemur immunocompetence, which is considered as an important proxy for their health. In

chapter 6, I evaluate the immunocompetence of multiple lemur species by analysing the allelic variation within the MHC II *DRB* region.

STUDY SITES, SPECIES, AND DESIGN

True lemurs (genus *Eulemur*, family Lemuridae) diverged 4.5 mya into twelve different species (Markolf and Kappeler, 2013; Yoder and Yang, 2004). *Eulemur* species are medium-sized (body and tail length 30-50 cm, 2-4 kg) and occupy a relatively broad range of Madagascar's remaining natural habitats (Andrainarivo et al., 2008; Mittermeier et al., 2008). Species within this genus are arboreal primates that occasionally move quadrupedally (i.e., on four legs on the ground). The diet of most *Eulemurs* primarily consists of fruits, flowers, and leaves (Markolf and Kappeler, 2013), although these lemurs are all capable of adding alternative food sources, such as fungi and invertebrates, to their diet. True lemurs show many similarities in morphology, diet preferences, and social behaviour, especially when compared to species of more distantly related genera (Markolf et al., 2013). However, populations and species across Madagascar may differ in activity patterns, social organisation, body size, and diet composition as an adaptation to the different environmental conditions they are exposed to (Sato et al., 2016).

First, I sampled three populations of the common brown lemur (*E. fulvus*) in Ankarafantsika National Park (NP) (16°25'S, 46°80'E), a site that is located on the western side of Madagascar and consists of dry deciduous forest; in Andasibe Mantadia NP and Mitsinjo, located on the eastern side of Madagascar (18°92'S, 48°42'E) and characterised by relatively wet rain forest; and on Nosy Tanikely (13°28'S, 48°14'E), an island in the north-east of Madagascar that is covered with tropical vegetation. Second, I sampled the black lemur (*E. macaco*) on two islands: Nosy Be (13°19'S, 48°14'E) and Nosy Komba (13°28'S 48°20'E) in the far north of Madagascar, islands that are covered with tropical rainforest. Third, I sampled the red-fronted brown lemur (*E. rufifrons*) at four different locations. Three of these locations consist of dry deciduous forest and are located on the western- and south-western side of Madagascar (Goodman & Benstead, 2005): Kirindy Forest (20°07'S, 44°67'E), Isalo NP (22°36'S 45°19'E), and Zombitse NP (22°52'S 44°40'E). I also sampled *E. rufifrons* in Ranomafana NP in southeastern Madagascar (21°16'S, 47°20'E), a site that contains tropical rainforest (Wright et al., 2012). In this location, I also sampled the fourth species, the red-bellied lemur (*E. rubriventer*). Relative to the humid eastern rainforests, western regions show pronounced seasonality, have a higher annual mean temperature, and less rainfall.

The main difference in social organisation between the different *Eulemur* species is their group size. *Eulemur fulvus*, *E. macaco*, and *E. rufifrons* live in multi-male, multi-female groups comprising four to eighteen individuals, whereas *E.*

rubriventer lives in small monogamous groups of two up to five individuals (Tecot, 2008, Tecot, 2010, Tecot et al., 2016). Two of these species (*E. rufifrons* and *E. rubriventer*) live sympatrically in Ranomafana NP but do not hybridise, and all other *Eulemur* populations are reproductively isolated.

Most of the chapters in this thesis focus on four different true lemur species (genus *Eulemur*) in ten different populations at nine different geographically separated locations across Madagascar (Table 1.2). As these species are widely distributed and occur in areas that experienced both low and high intensity logging, this provided the unique opportunity to identify the impacts of various natural and anthropogenic challenges to lemurs in more intact as well as disturbed habitats, and across diverse biogeographic regions.

One of the rainforest sites, Ranomafana NP, experienced varying levels of logging in the past (Mittermeier et al., 2008; Overdorff, 1993, 1996). This variation in logging history allows for a natural experiment to study lemur populations living across a (historical) logging gradient (Wright et al., 2005). Here, I could test for potential effects of such anthropogenic disturbances on forest structure and composition, as well as lemur presence and coexistence patterns as a consequence of potential forest alterations.

Eulemur rufifrons and *E. rubriventer* live in sympatry with five other more distantly related diurnal lemur species within Ranomafana NP: the black-and-white ruffed lemur (*Varecia variegata editorum*), Milne-Edwards' sifaka (*Propithecus edwardsi*), grey bamboo lemur (*Hapalemur griseus ranomafanensis*), golden bamboo lemur (*Hapalemur aureus*), and greater bamboo lemur (*Prolemur simus*) (Wright and Andriamihaja, 2002). To evaluate how these species cope with the effects of previous logging, I recorded the encounter rates and cluster sizes of these diurnal species within this lemur community at five sites with high and low levels of human disturbance. I also recorded the presence and behaviour of *E. rufifrons* and *E. rubriventer* to explain how these closely related species can coexist in this forest.

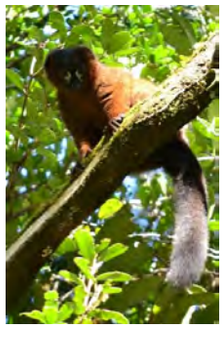
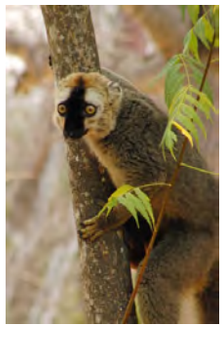
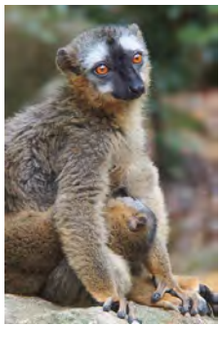
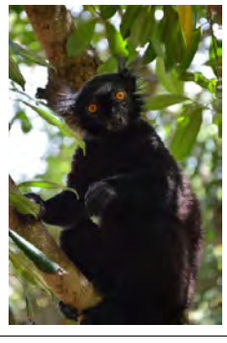
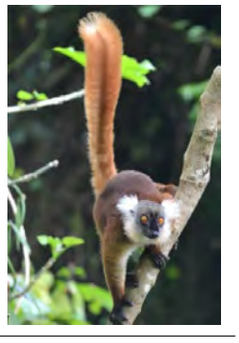
OVERVIEW

In this thesis, I explore the relationship between natural and anthropogenic impacts on the abundances and health of multiple lemur species across Madagascar. By integrating non-invasively collected field data, I quantified forest structure variables and characterised forest composition, performed transect surveys to determine lemur encounter rates and cluster sizes, and collected behaviour data as well as faecal and hair samples. The latter were used to sequence faecal bacterial microbiota, to morphologically identify parasite species, and to analyse MHC II diversity. My thesis consists of two parts: in the first part, I link the effect of anthropogenic disturbances to forest structural changes, lemur encounter rates, and lemur coexistence; in the

second part, I explore the health responses of lemurs to anthropogenic disturbances and environmental challenges. In the synthesis, I discuss other challenges that biodiversity conservation in Madagascar will face in the near future, and provide potential solutions to ensure the long-term survival of lemurs.

In **chapter 2**, I describe the impact of selective logging on forest structure and composition, as well as encounter rates and cluster size of multiple sympatric diurnal lemur species at sites that have experienced different logging intensities in the past. I discuss whether logged forests have recovered to pre-logging conditions, and consider the potential for regenerating forests to support lemur communities. Next, in **chapter 3**, I link the effect of anthropogenic disturbances to lemur coexistence. I focus on the stable coexistence of two congeneric and sympatric lemur species that share many ecological characteristics. The chapter contains the results of a quantitative behavioural study of habitat selection by and direct competition between both species. I evaluate whether potential niche differences (i.e., in diet overlap, spatial patterns, and temporal activity), interaction patterns between species, and reaction to forest disturbance can explain the coexistence of these closely related lemur species. In **chapter 4**, I provide an explorative assessment of the most important features of the faecal bacterial microbiota composition in different lemur species. I address to what extent geographic location, lemur species, sex, and age influence the intestinal microbial composition, and what factor contributes most to intestinal microbiota differentiation. **Chapter 5** focuses on nematode prevalence and faecal microbial composition in different lemur species occupying varying habitats. I evaluate the main drivers of both these GI inhabitants (i.e., nematodes and microbiota), and test for the effect of geographic location, seasonality, and anthropogenic disturbances. I also examine potential interactive effects between GI nematodes and microbiota. In **chapter 6**, I provide a comparative study on the immunocompetence of lemurs by analysing the allelic variation and allele polymorphism in the second exon within the MHC II *DRB* region in multiple *Eulemur* species and populations. For this chapter, a new primer set was developed that amplifies nearly all polymorphic codons (resulting in amino acid variation) of the antigen-binding site. I provide the level of sequence and functional polymorphism, and identify potential gene duplications, allele sharing between populations, and balancing selection. In the final chapter, **chapter 7**, I synthesise the results of my thesis by connecting the major insights from the diverse chapters, discussing the limitations and implications of my work, and suggesting future research directions. I also consider other challenges that biodiversity conservation in Madagascar is facing in the near future, and propose potential solutions to ensure the long-term survival of lemurs.

Table 1.2: *Eulemur* species. Common and Latin names of the four *Eulemur* species sampled for this thesis, sample location, and pictures of male and female individuals *(pictures taken by I. de Winter)*.

Species names	Sample location(s)	Male	Female
Common brown lemur *Eulemur fulvus*	Andasibe Mantadia NP and Mitsinjo, Nosy Tanikely, Ankarafantsika NP		
Red-bellied lemur *Eulemur rubriventer*	Ranomafana NP		
Red-fronted brown lemur *Eulemur rufifrons*	Ranomafana NP, Zombitse NP, Isalo NP, Kirindy Forest		
Black lemur *Eulemur macaco*	Nosy Be, Nosy Komba		

CHAPTER 2

ANTHROPOGENIC DISTURBANCE EFFECTS REMAIN VISIBLE IN FOREST STRUCTURE, BUT NOT IN LEMUR ABUNDANCES

de Winter, I.I., van der Hoek, S., Schütt, J., Heitkönig, I.M.A., van Hooft, W.F., Gort, G., Prins, H.H.T., Sterck, F.

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ABSTRACT

The persistence of tropical rainforests, together with their flora and fauna, is highly threatened by anthropogenic disturbances. In this study, we investigate to what extent selective logging influences the structure and composition of a tropical rainforest in Madagascar and subsequently lemur encounter rates and cluster sizes. We quantified forest structure variables and conducted transect surveys of seven sympatric diurnal lemur species in five protected forest sites with different logging histories. We found that tree species, family richness, DBH, tree height, and interquartile ranges of DBH and tree height (measure of forest heterogeneity) were relatively high and tree density relatively low in less disturbed compared to disturbed sites. Although the disturbed forests have not fully recovered to previous conditions, from a functional perspective, they seem to have recovered into suitable lemur habitat, as lemur encounter rates and cluster sizes were similar in disturbed and less disturbed sites. We only found slightly higher encounter rates for *Varecia variegata* ($P = 0.078$) and lower encounter rates for *Eulemur rufifrons* ($P = 0.059$) in less disturbed forests. This is one of the first studies that reports the presence of *V. variegata*, a species characterised by its drastic decline, in previously logged sites. Lemurs travelling between disturbed and less disturbed sites disperse seeds and hereby facilitate forest regeneration. Therefore, we promote the need for better attention to the value of logged forests for biodiversity conservation in Madagascar and suggest that there is considerable potential for regenerating logged forests to support lemur communities.

INTRODUCTION

Tropical forests hold most of the earth's terrestrial biodiversity, as they provide habitat for a vast array of plants and animals, and provide important ecosystem services, such as nutrient cycling, soil formation, and water retention (Gardner et al., 2009; Laurance, 2015). An increasing number of tropical forests have been disturbed by human activities, such as deforestation, logging and fragmentation, and by the consequences of anthropogenic climate change (Bradshaw et al., 2008). These human-induced changes can disrupt forest stability and can have long-lasting impacts on forest structure and biodiversity (Laurance, 2015). In particular, intensive logging drastically changes the forest structure and can result in a structurally more homogeneous forest canopy (DeWalt et al., 2003). Fragmented or homogenous tropical forests show a reduced diversity of mammal species (McElhinny et al., 2005; Michalski et al., 2007; Pardini et al., 2005) and are therefore a primary concern for conservation scientists and practitioners worldwide (Michalski et al., 2007; Ogrzewalska et al., 2011).

The tropical rainforests of Madagascar are among the most biodiverse habitats on earth and are widely considered a global conservation priority (Brooks et al., 2006; Ganzhorn et al., 2014; Myers et al., 2000). Madagascar experiences high rates of deforestation and ongoing habitat loss (Scales, 2014), hereby threatening many endemic species, including lemurs, with extinction (Goodman and Jungers, 2014; Schwitzer, 2014). Many of the fruit trees important for lemur species' survival are hardwood species favoured by selective loggers, and thereby, logging affects frugivore lemur populations (Wright et al., 2005). Nevertheless, not all lemur species are similarly affected by selective logging. Like in other primate (e.g., Johns and Skorupa, 1987) as well as non-primate species (Bicknell and Peres, 2010), lemurs characterised as folivores generally show less negative responses to such disturbances compared to frugivores (Herrera et al., 2011; Lehman et al., 2006a). Bamboo is the almost exclusive food source for bamboo lemurs (Yamashita et al., 2009), and is typically present in the dense understory of disturbed Malagasy forests in the proximity of human settlements (Olson et al., 2013). Although some lemur species seem to cope with human-induced forest alternations, habitat loss and fragmentation are among the most pervasive causes of declining lemur populations and biodiversity loss in Madagascar (Gardner et al., 2010; Irwin et al., 2010; Laurance et al., 2000).

In selectively logged forests, large mature trees of commercial value are usually removed (McElhinny et al., 2005). For that reason, past logging can result in a lower average tree diameter, tree height and crown volume, and higher tree densities due to the increased emergence of tree saplings (Gibson et al., 2011). The removal of such high-canopy trees also creates profound gaps in the upper forest canopy, thereby reducing canopy closure and consequently increasing light and reducing humidity (Van den Meersschaut and Vandekerkhove, 2000). Within primate communities,

species can react differently to such changes in habitat conditions. Primate species with specialised diets and characterised by slow reproduction are most affected by forest disturbances compared to more generalist species (Cowlishaw et al., 2009). In addition, small-scale disturbances can be beneficial for both folivorous and frugivorous species, as fruit production and leaf quality increase following elevated sunlight exposure at the understory level (Ganzhorn, 1995). Furthermore, logging can lead to changes in forest heterogeneity (Swanson et al., 2011). Numerous studies have found a positive relationship between increasing heterogeneity in multiple forest characteristics after logging events and the diversity in potential habitats, essential food resources, and shelter for animal communities (e.g., Dunn, 2004; Yang et al., 2015). Heterogeneity in forest structure is associated with a high degree of biodiversity (McElhinny et al., 2005). The relations between logging, forest structure and primate densities are complex and reflect varying causalities (Lehman et al., 2006a). Anthropogenic disturbances typically reduce species diversity, but species responses to disturbance are poorly known and can be very different across ecoregions (Irwin et al., 2010). It is important to have a local understanding of the responses of forests and lemurs to disturbances for conservation actions, for example in defining new protected areas. Therefore, we aim to assess the variation in Malagasy rainforest structure at sites that have experienced different logging intensities in the past and link these differences to the encounter rates and cluster sizes of multiple sympatric lemur species.

Other studies have addressed both structural and compositional changes in forests and at the same time linked these changes to primate abundances (Herrera, 2016; Lehman, 2007; Lehman et al., 2006b). For example, in West Malaysia, frugivore and folivore primates slowly recovered after disturbance, as logging drastically reduced overall food availability (Johns, 1988). In West Kalimantan, logged areas had fewer large food trees and a greater number of canopy gaps, leading to reduced orangutan (*Pongo pygmaeus*) nests (Felton et al., 2003). Furthermore, nocturnal lemur encounter rates were higher in primary compared to disturbed forests (Sawyer et al., 2017). In contrast, Javan slow lorises (*Nycticebus javanicus*) showed high abundances in agricultural mosaic habitats (Rode-Margono et al., 2014). Also in a dry deciduous forest in western Madagascar, lemur sightings of both folivore and frugivore species increased compared to the pre-logging state of the forest (Ganzhorn, 1995). In Ganzhorns' study, small scale disturbances created gaps in the forest canopy, which increased sun exposure for some trees, leading to higher fruit production and protein concentration in leaves. However, results were not consistent across years and some lemur species were probably not present during sampling due to hibernation. In addition, the increased sighting rate post-logging could be due to greater visibility through the vegetation (Ganzhorn, 1995). In a study on lemur density in response to

human disturbances, it was found that many lemur species responded positively to such disturbances, despite the negative influence on lemur food trees (Lehman et al. 2006). This study suggests that due to the lemurs' tolerance for human disturbances, some lemur species can survive the extreme habitat loss and forest fragmentation throughout Madagascar. In conclusion, these previous studies revealed no clear patterns in primate abundances as a result of structural and compositional forest changes.

The distribution and quality of food patches can influence the size of primate social groups (Chapman and Chapman, 2000). The crown volume of trees in relatively less disturbed forests is usually larger than in disturbed forests (Balko and Underwood, 2005; Tecot, 2008), influencing the availability of resources. The smaller and more widely dispersed food patches in disturbed forests can lead to increasing travel costs, especially for relatively large primate groups (Chapman and Chapman, 2000; Majolo et al., 2008). Therefore, some primate species lower their group sizes in disturbed areas to reduce these costs as well as the costs of within-group competition (Chapman and Chapman, 2000; Isbell, 1991).

In this study, we hypothesise that lemur abundance and cluster size vary according to disturbance-induced forest structure characteristics. These disturbances are expected to result in smaller trees, higher stem densities, lower heterogeneity in tree height and diameter, as well as a lower diversity in tree species and families. In particular, we predict: (1) that for *Varecia variegata*, the association between encounter rate and tree size, reflecting lower disturbances, is positive because of the species' specialised frugivorous diet; (2a) that the smaller, more generalist (i.e., *Eulemur rufifrons* and *E. rubriventer*) and (2b) the more folivorous lemur species (i.e., *H. griseus*) in our study are encountered at a higher rate in forests with smaller trees compared to the forests with larger trees; (3) and finally, that lemur cluster size among our study species increases in forests with larger trees that reflect a low disturbance history. To determine the impact of selective logging on lemur communities, we quantified forest structure variables and conducted transects surveys to determine the encounter rates and group sizes of seven sympatric diurnal lemur species (i.e., *Varecia variegata editorum, Eulemur rubriventer, Eulemur rufifrons, Propithecus edwardsi, Hapalemur griseus ranomafanensis, Hapalemur aureus*, and *Prolemur simus*) in five protected forest sites in Ranomafana National Park (NP), Madagascar, that have been subjected to different intensities of logging in the past.

METHODS

STUDY AREA AN SPECIES

This study was conducted in Ranomafana National Park NP (43,500 ha), a mid-altitude rainforest (600 to 1500 m), providing essential habitat to at least twelve species of Strepsirrhine lemurs in southeast Madagascar, located within the following coordinates: 20°58'22"S, 47°26'13"E, 20°27'25"S, 47°23'5"E, 21°8'23"S, 47°35'32"E and 21°15'45"S, 47°17'54"E) (Wright et al., 2012; Wright and Andriamihaja, 2002). Before the establishment of the National Park in 1991, the forest was subjected to different logging schemes, ranging from intensive commercial logging to gradual wood extraction for local subsistence (Wright and Andriamihaja, 2002). Ranomafana NP currently comprises a mix of pristine, nearly pristine, and regenerating rainforest. For this study, we selected five sites (ca. 1.5 km^2 each) within the park, where trails existed for following animals. These sites had been subjected to different intensities of anthropogenic disturbances nearly three decades ago and have been regenerating since (Wright et al., 2012; Wright and Andriamihaja, 2002). In our three disturbed sites, Talatakely (TALA), Sakaroa (SAKA), and Vohiparara (VOHI), extensive clearing for agriculture and human habitation in the 1950s was followed by intensive commercial logging until the late 1980s. Much of the secondary growth is dominated by dense stands of introduced Chinese guava (*Psidium cattleyanum*) as well as clumps of giant bamboo (*Cathariostachys madagascariensis*). In our less disturbed sites: Vatoharanana (VATO) and Valoahaka (VALO), commercial logging occurred in both sites, albeit with much lower intensity than in the disturbed sites. However, many rosewood (*Dalbergia* spp.) stumps are present in these latter sites, indicative of past logging (Balko and Underwood, 2005; Herrera et al., 2011; Wright et al., 1997) (Fig. 2.1; Table 2.1). We focused on all seven diurnal lemur species within the park: *Varecia variegata editorum, Eulemur rubriventer, Eulemur rufifrons, Propithecus edwardsi, Hapalemur griseus ranomafanensis, Hapalemur aureus*, and *Prolemur simus* (Wright and Andriamihaja, 2002) (Table 2.2).

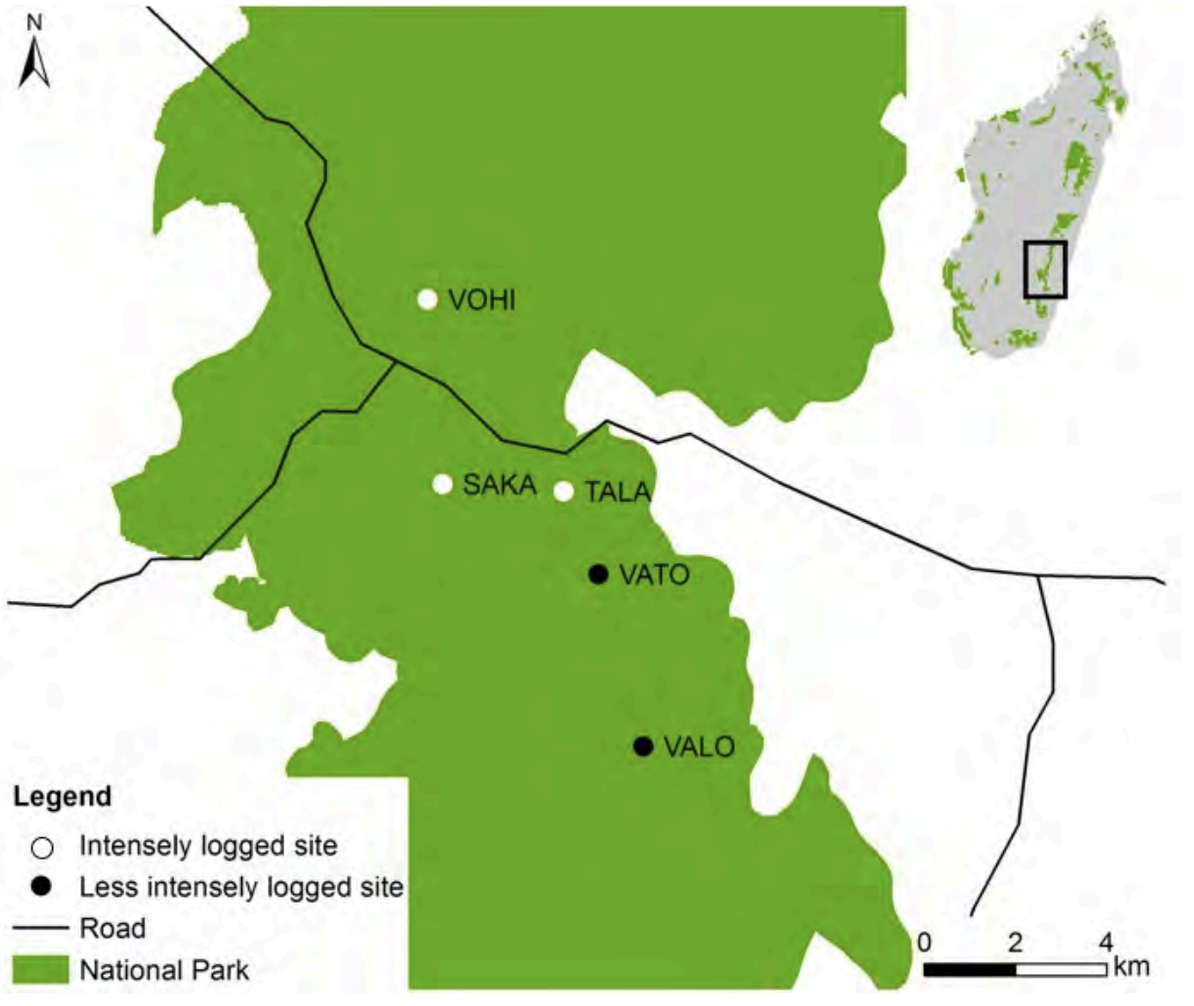


Figure 2.1: Map of Ranomafana National Park and the five research of this study. Three sites (white dots) experienced relatively intense logging in the past, while two sites (black dots) experienced no such disturbances. This map was generated via ArcGIS version 10.5. Data were downloaded from UNEP-WCMC and IUCN (2016), Protected Planet: National Parks of Madagascar; The World Database on Protected Areas (WDPA) [On-line], [May 2016], Cambridge, UK: UNEP-WCMC and IUCN.

Table 2.1: Overview of multiple disturbance parameters for the five research sites within Ranomafana National Park, Madagascar.

Research site	Logging intensity	Old rice paddies	Tourists	Research	Invasive plants	Elevation (m)	GPS S	GPS E
Sakaroa[a]	High	Yes	Little	Little	Chinese guava, *Psidium cattleianum*	1020	21°16′34″S	47°23′49″E
Talatakely[b]	High	No	Yes	Yes	Chinese guava, *Psidium cattleianum*	945	21°15′40″S	47°25′14″E
Vohiparara[c]	High	Yes	Yes	Yes	Chinese guava, *Psidium cattleianum*	1080	21°13′39″S	47°23′33″E
Vatoharanana[d]	Low	No	Little	Yes		995	21°17′33″S	47°25′41″E
Valohoaka[d]	Low	No	Little	Yes		995	21°18′78″S	47°26′25″E

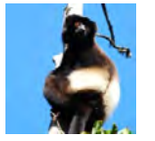
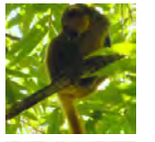
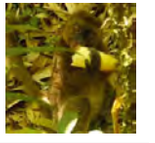
[a]Irwin et al., 2009

[b]Wright et al., 1997

[c]Andriamaharavo et al., 2010

[d]Balko and Underwood, 2005; Wright, 2009

Table 2.2: Diurnal lemurs present in Ranomafana National Park. Information on feeding guild, diet, body mass, and group size[a].

Common name	Scientific name	Feeding guild	Diet	Body mass (g)	Group size	Picture
Black-and-white ruffed lemur	*Varecia variegata editorum*	Frugivore	Fruits (90%), rest leaves	3650	2 to 5	
Red-bellied lemur	*Eulemur rubriventer*	Frugivore	Fruits (70%), rest leaves	2200	2 to 5	
Red-fronted brown lemur	*Eulemur rufifrons*	Frugivore	Fruits (70%), rest leaves	2000	4 to 18	
Milne-Edwards' sifaka	*Propithecus edwardsi*	Mixed diet	Fruits (30%), seeds (35%), leaves (28%)	5800	3 to 9	
Grey bamboo lemur	*Hapalemur griseus ranomafanensis*	Folivore	Bamboo (80%), rest fruit and leaves	935	3 to 5	
Golden bamboo lemur	*Hapalemur aureus*	Folivore	Bamboo (80%), rest fruit and leaves	1550	2 to 6	
Greater bamboo lemur	*Prolemur simus*	Folivore	Bamboo (95%), rest fruit	2450	4 to 7	

[a]Tan, 1999; Wright, 2006; Wright et al., 2008, 2005. *(Pictures taken by I. de Winter in Ranomafana National Park.*

FOREST STRUCTURE

We quantified forest structure variables at each site between April and June 2013. North-South surveys (*N* = 7 to 9 per site) were systematically established 150 m apart after randomly locating the first transect. For all forest measurements, we used line transects to randomly sample the habitat. Every 12.5 m, we set a sampling point centred on the transects following the point-centred quarter method (Cottam and Curtis, 1956). Four quadrants were formed by the transect line and its perpendicular and we selected the nearest tree in a quadrant. We aimed for 20 sampling points per transect but were sometimes forced to shorten transects due to landscape features (e.g., large rivers or cliffs). The total number of sampling points within a site was always equal (*N* = 140 sampling points, 560 trees). We determined the distance of the point centre to the nearest tree in a quadrant (DTPC) and identified the tree species. By using a measuring tape, diameter at breast height (DBH in cm) was determined at 1.30 m for all selected trees when DBH was > 5 cm. At each sampling point, canopy closure (10% - increments) was estimated using a spherical densiometer and elevation was determined by means of a Garmin *e*Trex Vista GPS receiver. Tree density was computed per sampling point: 10,000 / (average DTPC per quadrant)^2 (Balko and Underwood, 2005; Cottam and Curtis, 1956). Heterogeneities of DBH, height, DTPC, density, and canopy closure were defined as the interquartile ranges (IQR) per transect. The variabilities captured in this way are partly due to variance-mean relationships: e.g., in sites with taller trees, the variability of height tends to be higher.

LEMUR SURVEY PROCEDURE

To assess the relationship between lemur abundances and forest structure, we conducted transect surveys between November 2014 and February 2015, the period of fruit availability in Ranomafana NP (Tecot, 2008; Wright et al., 2005). For all the lemur measurements, we walked two-km long transect survey routes that followed the pre-existing trail system wherever possible to minimise forest impacts and to allow for more extensive surveys (Herrera et al., 2011; Hiby and Krishna, 2001; Lehman et al., 2006; Wright and Andriamihaja, 2002). We used curved line transects that we call 'transect surveys' and define a transect as a path along which one counts and records occurrences of the species of interest (Hiby and Krishna, 2001). The trail system in our sites was originally established for the explicit purpose of following known groups of animals. We walked the survey transects in a team of one observer and one assistant, trained at the Centre ValBio research station, at a slow, constant pace of one km/h. For each visual lemur encounter we recorded the species observed, social cluster size, and the presence of inter- or intra-specific social clusters of lemurs. We consider our encounters to be with clusters of lemurs instead of complete social groups, as we cannot assume that the entire social group is encountered at once (Plumptre and Cox,

2006). Some individuals may be at the periphery of the observed cluster and allude detection. In addition, some species have dispersed foraging patterns resulting in large group spreads, which would again lead to an underestimation of group size. Individuals of a specific species found < 50 m of each other along a transect survey were considered to be in the same cluster. As a proxy for abundance, we calculated lemur encounter rates (number of lemur cluster encounters/km) and we counted the number of lemurs per cluster (Herrera et al., 2011). Three non-overlapping transect surveys per site (total: 15 different survey routes) were established and repeatedly (four to ten times) surveyed. The time and starting point was alternated in a way that transect surveys were not measured more than once every 18 hours (Herrera et al., 2011). Each day, we walked three transect surveys at different times of the day: < 09:00 h, 09:00-12:00 h, and > 12:00 h. When weather conditions reduced the visibility to less than 15 meters, no survey was conducted. This transect survey method is well established and routinely accomplished for different forest species (Herrera et al., 2011; Irwin et al., 2005; Johnson and Overdorff, 1999; Lehman, 2007). Per survey, the number of encounters with lemur groups and their group sizes were scored for each lemur species. Based on a total search effort of 210 km, this led to a dataset with 89 actual lemur encounters at five sites, three different transects per site, four to ten surveys per transect, yielding 19 to 23 transect surveys per site. We compared species richness between sites by rarefaction (Colwell et al., 2012). To visualise the sampling effort per site we used EstimateS version 9.1.0 (Colwell, 2013). Rarefaction was conducted with 100 randomisations using EstimateS and Standard Deviations were depicted for each curve (supplementary material, available online).

STATISTICAL ANALYSIS

We compared forest structural characteristics on three hierarchical levels among the five sites. 1) At tree level, DBH, tree height, and DTPC were obtained; 2) at sample point level, we obtained values for canopy closure and tree density; 3) at transect level (each including 10 to 20 sampling points), forest heterogeneity measurements (IQR), tree species richness and family richness, were obtained. For the analysis of the forest characteristics at the lowest (tree) level, we used linear mixed models (LMM; Zuur et al. 2009) with fixed effects for research sites and random effects for transects and sampling points within transects. For the analysis of characteristics at the intermediate (sampling point) level, mixed linear models with fixed effects of sites and random effects of transects were used. For the analysis of characteristics at the highest (transect) level, ordinary one-way ANOVA was used to compare sites. Based upon residual analysis, we used power transformation responses where needed to achieve approximate normality and variance homogeneity of error distributions (Zuur et al., 2009): log(DBH4.5), $\sqrt{height}$, $\sqrt[3]{DTPC}$, $\sqrt{100 - canopyopeness}$, log(density), and

log(IQR height). Transformed height was found to have unequal variances among sites, which was accommodated for in the LMM. Sites were compared with approximate *F*-tests, using the method according to Kenward and Roger for calculating degrees of freedom (Kenward and Roger, 1997) in the LMM's, or with regular *F*-tests (in ANOVA), followed by post-hoc pairwise comparisons among sites using Tukey Honestly Significant Difference (HSD) correction to *P*-values (Tukey, 1949). Furthermore, 95% confidence intervals for the mean response per site were calculated and shown in bar diagrams or tables. To compare previously disturbed and less disturbed sites, the means for the disturbed (TALA, SAKA, and VOHI) and for the less disturbed sites (VATO and VALO) were estimated and compared with *Z*-tests (in LMM) or *t*-tests (in one-way ANOVA).

Regarding the lemur distribution across sites, we first gave descriptive statistics. Next, to test for differences in lemur encounter rates (number of clusters per transect survey) and cluster sizes, we used generalised linear mixed models (GLMM) using the Poisson distribution and log link (Bolker et al., 2009), with fixed effects for starting time, species, forest disturbance, and species by disturbance interaction, and with random effects for site, species by site interaction, transects, and surveys. We analysed the average cluster size per survey, using the total count per survey as response variable and log(encounter rate) as offset. Models were compared using likelihood ratio tests, but also using AICc values (Symonds and Moussalli, 2011). We also included Nakagawa and Schielzeth's pseudo R^2 statistics, as available in R's MuMIn package (Nakagawa and Schielzeth, 2013). We checked all models containing subsets of fixed effects and report the models ranked by AICc criterion (Supplementary Material, available online). Depending upon overall results, specific user-defined contrasts were studied, using *Z*-tests (Wald tests), and predicted means with 95% confidence intervals were calculated. Here, we excluded the bamboo lemur species *H. aureus* and *P. simus* due to very low sample sizes. Statistical analyses were performed using R (version 3.4.1, R CoreTeam, 2017) and R-package lme4 (version 1.1-1.3, Bates et al., 2015) with add-on packages for testing and prediction (lmerTest version 2.0-33, pbkr version 0.4-7; lsmeans version 2.27-2), for user-defined contrasts (multcomp version 1.4-7), and multimodel inference (MuMIn version 1.15.6 9).

RESULTS

FOREST STRUCTURE

The following forest structural characteristics differed among sites: DBH, tree height, DTPC (Fig. 2.2), and tree density. The five forest sites surveyed showed significant differences in DBH ($F_{4,33.0}$ = 24.82, P < 0.001, Fig. 2.2A). DBH was highest in the less disturbed compared to the disturbed sites (back-transformed means: 12.6 cm and 9.8 cm respectively, Z = 8.19, P < 0.001). In the less disturbed sites, > 7% (VALO) and > 9% (VATO) of the stems exceeded a DBH of 40 cm (median = 12.9 cm, Q1-Q3: 8.6 cm - 23.5 cm), while trees in sites that have been subjected to intensive logging rarely exceeded diameters of 40 cm (VOHI: 1.4%, TALA: 1.5%, and SAKA: 3%, median = 10.0 cm, Q1-Q3: 7.2 cm - 15.8 cm). The five forest sites showed significant differences in tree height ($F_{4,34}$ = 7.78, P < 0.001, Fig. 2.2B). Trees were significantly taller in the less disturbed sites compared to the disturbed sites (back-transformed means: 10.7 m and 9.2 m respectively, Z = 5.08, P < 0.001). The proportion of high-canopy trees (> 20 m) in the less disturbed sites, VATO and VALO, was > 9% (median = 11 m, Q1-Q3: 7 m - 16 m), whereas in the highly disturbed sites < 4% (VOHI: 0.2%; TALA: 1.4%, and SAKA: 4%) of all trees exceeded heights of 20 m (median = 9 m, Q1-Q3: 6 m - 12 m). DTPC differed among forest sites ($F_{4,33.1}$ = 11.71, P < 0.001, Fig. 2.2C) and was significantly highest in the less disturbed sites compared to the disturbed sites (back-transformed means: 2.1 m and 1.7 m, respectively, Z = 5.08, P < 0.001). Tree density also differed among sites ($F_{4,33.1}$ = 11.26, P < 0.001) and was significantly lowest in the disturbed sites compared to the less disturbed sites (back-transformed means: 2028 and 2921 trees per hectare, respectively, Z = -4.87, P < 0.001). We found no significant differences in canopy closure when comparing all sites ($F_{4,33.1}$ = 1.93, P = 0.128, Fig. 2.2D) and when comparing the previously disturbed versus less disturbed sites (Z = -1.45, P = 0.146, back-transformed means 70.7% and 73.3% respectively).

Tree species and family richness were both significantly different among sites ($F_{4,33}$ = 9.54, P < 0.001 and $F_{4,34}$ = 5.53, P = 0.002 respectively), with a higher diversity in the less disturbed compared to the disturbed sites (back-transformed means: tree species richness: 39.2 and 32.0 different species per transect survey respectively, t_{34} = 4.29, P < 0.001; tree family richness: 23.3 and 20.7 different families per transect survey respectively, t_{34} = 2.87, P = 0.007, Fig. 2.3A, B).

The Inter Quartile Range of DBH, tree height, DTPC, and tree density were significantly different among sites, while canopy closure did not show statistical differences (supplementary material, available online). DBH, height, and DTPC were more variable whereas tree density was less variable in less disturbed than in disturbed sites.

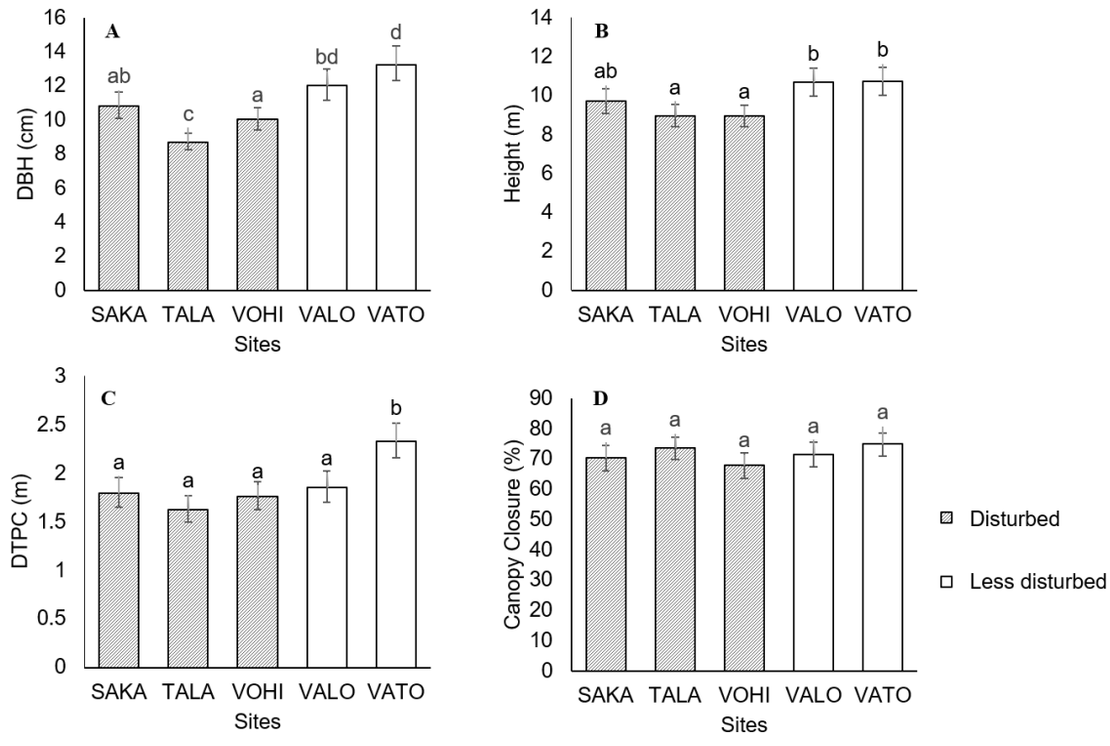


Figure 2.2: Forest structural characteristics. Mean A) DBH (cm); B) height (m); C) distance of the point centre to this nearest tree (DTPC) (m); D) and canopy closure (%) with 95% confidence intervals measured in five study sites with different disturbance histories in Ranomafana National Park. The bars with letters in common do not differ significantly in post-hoc tests.

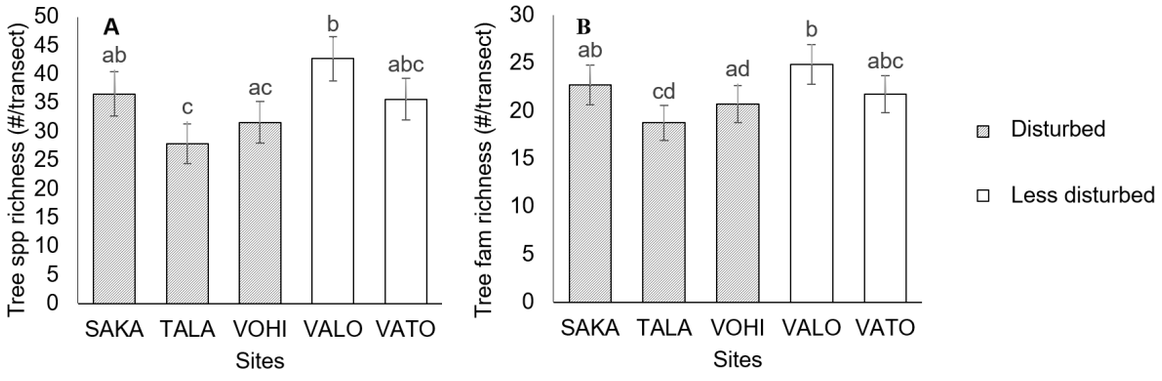


Figure 2.3: Mean A) tree species and B) tree family richness with 95% confidence intervals measured in five study sites with different disturbance histories in Ranomafana National Park, Madagascar. The bars with letters in common do not differ significantly in post-hoc tests.

LEMUR ENCOUNTER RATES

A total of 351 (346 after excluding the two rare bamboo lemur species) lemur individual and 89 (86 after excluding the bamboo lemurs) cluster encounters were registered for seven different diurnal species across the five different forest sites. The overall model (GLMM), containing fixed effects for species, disturbance, and their interaction and starting time of the survey as covariate, was significant (LRT $\chi^2(11)$= 22.44, P = 0.021). A significant interaction between the encounter rates of the lemur species and forest disturbance was found (LRT $\chi^2(4)$ = 10.52, P = 0.032). The AICc of this model with interaction was 493.0 compared to 495.1 for the model without interaction, indicating too that not all five species had the same difference in encounter rates between disturbed or less disturbed forests. No significant difference in lemur encounter rates, averaging over species, was found between disturbed and less disturbed sites (LRT $\chi^2(1)$ = 0.96, P = 0.33). The model with lowest AICc value, however, was the model with starting time as the only fixed factor (AICc = 491.7 versus 493.0 for the full model). Focusing on individual lemur species we found that *V. variegata* and *E. rufifrons* showed slight, but not significant, differences between disturbed and less disturbed sites. The largest, most obligate frugivorous species, *V. variegata*, showed slightly, but not significantly, higher encounter rates in less disturbed compared to disturbed forest sites (0.07 versus 0.02 clusters per km survey transect, Z = -1.76, P = 0.078), while *E. rufifrons* showed slightly higher encounter rates in previously disturbed compared to less disturbed sites (0.11 versus 0.04 respectively, Z = 1.89, P = 0.059). The other three lemur species did not show any clear differences. For *E. rubriventer* the back-transformed means were 0.08 in disturbed and 0.11 in less disturbed sites (Z = -0.74, P = 0.46). For *P. edwardsi* we found back-transformed means of 0.08 in disturbed and 0.09 in less disturbed sites (Z = -0.17, P = 0.87). Finally, for *H. griseus*, the mean back-transformed encounter rates were 0.02 in disturbed and 0.06 in less disturbed sites (Z = -1.54, P = 0.12, Fig. 2.4).

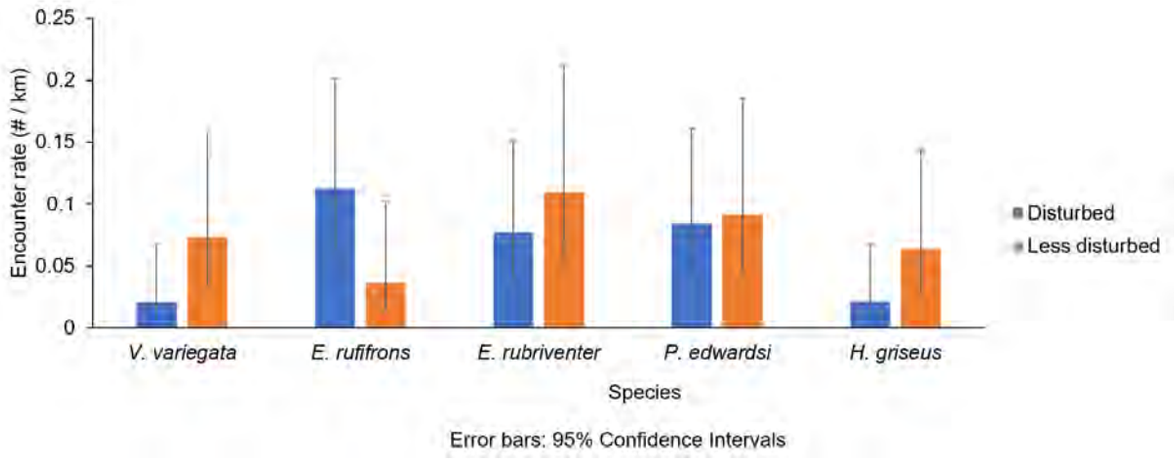


Figure 2.4: Lemur cluster encounter rates (#/km) with 95% confidence intervals measured in disturbed and less disturbed sites in Ranomafana National Park, Madagascar. The large confidence intervals are likely the result of the variability in encounter rates among transects within sites and most likely related to low sample sizes.

CLUSTER SIZE

The range of observed cluster sizes varied from 1 to 12 individuals. No effect of starting time of the survey was found and it was therefore removed from the model (LRT $\chi^2(2) = 0.71$, $P = 0.70$; AICc values with and without starting time were 345.0 and 339.5). No significant interaction between lemur species and forest disturbance was found (LRT $\chi^2(4) = 1.65$, $P = 0.20$; AICc values with and without interaction were 339.5 and 331.1). Cluster sizes differed significantly between lemur species ($\chi^2(4) = 34.1$, $P < 0.0001$; AICc values 331.1 and 355.1), with the largest cluster size for *E. rufifrons*. No difference in cluster size between disturbed and less disturbed forest sites was found (LRT $\chi^2(4) = 1.7$, $P = 0.20$; AICc values 331.1 and 330.1). Focusing on individual species, we found that only *V. variegata* showed a slightly, but not significantly, larger cluster size in previously disturbed sites compared to less disturbed sites ($Z = 1.88$, $P = 0.059$, back-transformed means 3.26 and 1.36 individuals per group respectively, Table 2.3).

Table 2.3: Mean cluster size of the five diurnal lemur species in Ranomafana National Park in the previously disturbed and in the less disturbed sites with 95% confidence intervals (CI).

	Disturbed		Less Disturbed			
Species	**Mean**	**95% CI**	**Mean**	**95% CI**	***Z***	**Pr (> |*Z*|)**
Varecia variegata	3.25	1.68 - 6.30	1.36	0.73 - 2.53	1.88	0.06
Eulemur rufifrons	8.77	7.35 - 10.45	7.08	4.81 - 10.41	0.99	0.32
Eulemur rubriventer	3.36	2.40 - 4.71	3.36	2.43 - 4.64	0.01	0.99
Propithecus edwardsi	3.90	2.89 - 5.26	3.48	2.46 - 4.93	0.49	0.63
Hapalemur griseus	2.90	1.45 - 5.81	2.80	1.76 - 4.45	0.09	0.93

DISCUSSION

FOREST STRUCTURE

As expected, our results showed several ecological differences in forest structure and tree species composition among the study sites. Even nearly 30 years after the last logging event (Wright and Andriamihaja, 2002), the forest sites that were exposed to intensive logging were characterised by smaller trees, a lower diversity in tree species and families, higher stem densities, and lower heterogeneity in tree height and diameter compared to the less disturbed sites. Our results reflect the impact of commercial exploitation on tropical rainforests and match successional patterns described elsewhere (Chazdon, 2014; Guariguata and Ostertag, 2001). After selective logging, changes in light penetration through the canopy can lead to fast recolonisation of plants (Asner et al., 2004). The less disturbed forests had relatively high, closed canopies, whereas disturbed forests had lower canopies, dense young understory trees, and experienced a canopy-closing impact of climbers and bamboo. The presence of this typical understory is also reflected in the higher tree densities we found in these disturbed sites. The apparent structural and compositional differences among forest sites within Ranomafana NP were also found in previous studies (Balko and Underwood, 2005; Brown and Gurevitch, 2004). But while Balko and Underwood (2005) found that canopy closure was significantly higher in VATO and VALO compared to TALA, we found no such difference in our study ten years later, likely due to further regeneration of the forest. Complete deforestation in Malagasy forests, such as in Menabe, can have irreversible effects due to low regenerative power and invasion by alien plants (Lowry et al., 1997). However, recovery of tropical rainforests after disturbance can be relatively rapid (Aide et al., 1995). Forest recovery is generally faster when disturbance primarily impact forest canopies and residual vegetation remains to promote seedling regeneration and reestablishment of original forest species (Chazdon, 2003; Guariguata and Ostertag, 2001). Remaining trees or shrubs may function as key sources for dispersal of seeds, particularly when appropriate dispersal agents are still present (Bleher and Böhning-Gaese, 2001; Duncan and Chapman, 1999; Lamb et al., 2005). Buried seeds in the soil are also an important contributor to regeneration, especially when disturbances are low to moderate and when forest soils have remained unaffected (Guariguata and Ostertag, 2001). Lastly, as an adaptation to recover from disturbances, many tropical rainforest trees have the ability to resprout after being damaged (Paciorek et al., 2000). The forests in Ranomafana NP have never been clear-cut on a large scale, with no significant disturbance of the forest soil and seedbanks and strict protection since 1991. This is probably one of the important factors explaining Ranomafana NP forests' capacity to regenerate. So, the forests of our study are not free of the effects of past disturbance

and have not returned to identical pre-logging conditions, but ecosystem functions seem to have returned to pre-disturbance levels (Gardner et al., 2007).

LEMUR ENCOUNTER RATES

Some studies question the value of previously logged forests, as such forest could be unsuitable habitat for specific forest species (Zinner et al., 2014). A recent survey at Ranomafana NP suggested indeed that some frugivorous lemurs were more abundant at a less disturbed site, when compared to a highly disturbed site (Herrera et al., 2011). Despite structural and compositional differences across the forest areas, however, we found similar lemur encounter rates across the different forest sites, which suggests that the lemur species can cope with the forest structural differences we found. For example, at all sites, the canopy has been quantified as rather closed, most likely facilitating the movement of the canopy-dwelling lemurs present. Many other forest functions may resemble undisturbed conditions long before tree species composition does (Guariguata and Ostertag, 2001). Concerning the ecosystem function of providing habitat to multiple lemur species, it seems that the forests of Ranomafana NP are approaching pre-logging conditions.

Although we hypothesised, and other studies found, that larger and more frugivorous species decrease with disturbance (Irwin et al., 2009), we only discerned a trend ($P = 0.078$) that the large-bodied, most obligate frugivore species in our lemur community, *V. variegata*, shows somewhat higher encounter rates in the less disturbed compared to the disturbed forest sites. Previous studies on the population densities of this species, of which some were long-term studies starting from the inauguration of Ranomafana NP, indicated with more certainty that the abundance of *V. variegata* was highest in the less disturbed sites and was low or even absent in the disturbed sites (Balko and Underwood, 2005; Herrera et al., 2011; Irwin et al., 2005; Johnson et al., 2003; Lehman et al., 2006a; Wright et al., 2012). Other studies on primate densities in different tropical forests also showed frugivore population densities to be affected by anthropogenic disturbance (Schmidt and Jensen, 2003). For instance, a long-term study on the effect of logging on African primate communities found that densities of frugivorous primates were still declining 28 years post-harvesting in sites that experienced selective logging (Chapman et al., 2000). Furthermore, it was found that heavily disturbed sites in tropical forests in Kenya and Uganda showed reduced species richness and densities of frugivorous bird and primate communities (Kirika et al., 2008). A long-term study would be necessary to determine if the groups of *V. variegata* that we encountered are resident to the disturbed site year-round, as the species may require access to less disturbed habitats for large mature fruit trees, shelter, or reproduction (Balko, 1998; Balko and Underwood, 2005). Nevertheless, our observations of the presence of *V. variegata* in the disturbed forest sites suggest that

ecosystem function is progressing towards pre-disturbance levels.

During our study, we regularly observed *V. variegata* feeding on Chinese guava (*Psidium cattleyanum*) and the fruit of this tree is utilised by several other lemur species as well, including *Eulemur rufifrons* and *E. rubriventer* (Birkinshaw and Colquhoun, 2003; Grassi, 2006; Overdorff, 1993; Razafindratsima et al., 2014). Chinese guava was introduced in 1947 when villagers were forced to move to the main road to settle there. The guava moved into the abandoned villages in all our previously disturbed sites (P.C. Wright, *personal observation* and C. Hooper, *unpublished report*). This introduced guava grows fast and produces fruit far more quickly than endemic fruit trees. We suggest that current feeding habits have expanded *V. variegata's* diet to the introduced Chinese guava. We put forward the possibility of temporal migration by *V. variegata*, with its large and seasonally variable territories (Balko and Underwood, 2005), to the disturbed sites, where these guava fruits are seasonally highly available.

As hypothesised, the relatively small and more generalist species *E. rufifrons* showed slightly higher encounter rates in the disturbed compared to the less disturbed forest sites ($P = 0.059$). Generalist species tend to increase when exposed to habitat disturbances, as these species are more flexible in their habitat use and tend to be more tolerant to human activities (Cameron and Gould, 2013; Chazdon, 2014; Gabriel, 2013; Ganzhorn et al., 2003; Johns and Skorupa, 1987; Pardini et al., 2009; Peres, 1994). Our results differ from a study performed shortly after the main logging events within the national park, where *E. rufifrons* occurred in greater densities in one of the less disturbed sites compared to a more disturbed site (Overdorff, 1991). Now, years after the last logging event, our results may be reflecting that *E. rufifrons* has expanded its range to the disturbed sites. So, this species with its highly flexible home ranges and temporary range shifts can persist in disturbed areas. However, it was also observed that groups of *E. rufifrons* were forced to migrate and travel 4-5 km from their usual home ranges to other areas that contained more abundant fruits and returned later (Overdorff, 1993, 1996). As a consequence, our results may not reflect long-term differences in abundance, but short-term movements of groups in response to resource availability.

Our results also show that the congeneric species *E. rubriventer*, another relatively small-bodied generalist, subsists in both disturbed and less disturbed habitats (Dehgan, 2003; Ganzhorn et al., 2003; Herrera et al., 2011), but the species did not show higher encounter rates in disturbed forests, as we hypothesised. Ten years ago, the abundance, quality, and predictability of fruit for this species was impaired, individuals were less active, and had higher infant mortality within the disturbed forest sites (Tecot, 2008). Another study on this species in Ranomafana NP revealed many behavioural similarities and overall diet breadth in disturbed and less disturbed sites (Durham, 2004). In sites or periods with limited or unreliable food

supplies, *E. rubriventer* may sacrifice reproduction for survival (Tecot, 2012). Hence, *E. rubriventer* is considered as a flexible species that shows behavioural plasticity, which makes the species more resilient to disturbance effects (Dehgan, 2003).

Encounter rates of *P. edwardsi* did not differ between the previously disturbed and less disturbed sites. However, ten years post selective logging, energy intake, and hence body weight, of *P. edwardsi* was significantly lower in the disturbed compared to the less disturbed forests (Wright et al., 2005). Logging may thus have consequences for this species' survival and reproductive success (Arrigo-Nelson, 2006). Other long-term studies have found that the population size of *P. edwardsi* did not change in sites with low disturbance but oscillated in sites with high disturbance (Pochron et al., 2004). Previous surveys in Ranomafana NP have also shown that lemur population sizes of multiple species can oscillate. For example, population densities of *V. variegata* and *P. edwardsi* have oscillated, likely in relation to fossa predation and food scarcity (Wright et al., 2012), while congeneric competition between *E. rufifrons* and *E. rubriventer* may have caused oscillations in these species population densities (Erhart and Overdorff, 2008; Johnson and Overdorff, 1999). *Propithecus edwardsi* is known as an opportunistic frugivore, tracking the fruit availability in the forest (Wright et al., 2005), and its abundances in different sites may therefore follow fruiting patterns within seasons and throughout the year. Thus, the species' variation in home range size (Morriss et al., 2009) and flexibility in the number of fruit species in its diet (Arrigo-Nelson, 2006) may enable *P. edwardsi* to live in the different sites of this study area.

Up to 90 percent of all tree species in tropical rainforests rely on animals for their seed dispersal (Jordano, 1992). Seed dispersing frugivores and granivores therefore play an important role in plant colonisation and forest restoration and regeneration (Jordano et al., 2011; Medellin and Gaona, 1999). Particularly in disturbed habitats, seed dispersers are important to bring forest tree species back and to initiate successional processes (Holloway, 2000). Malagasy forests show a low richness of frugivore communities and here, lemurs play an important role in seed dispersal (Hawkins and Goodman, 2003; Razafindratsima and Dunham, 2015). Hence, the regeneration of forests with the complete set of primary forest tree species can depend on the presence of seed-dispersing lemurs (Ganzhorn et al., 1999). Large-bodied frugivores, including *V. variegata,* are the predominant seed dispersers of large fruit trees (Markl et al., 2012; Razafindratsima et al., 2014) and travelling from relatively less disturbed to disturbed forest sites has important consequences for forest recovery. So, the migrant behaviour of *V. variegata* could be crucial in the re-population of endemic fruit trees in a previously disturbed forest. *Eulemur rufifrons* and especially *E. rubriventer* are also known as important seed dispersers who increase tree recruitment probability in this system (Razafindratsima and Dunham,

2015) and the presence of these lemur species in disturbed areas suggests that the seed dispersal that these lemurs perform is maintained. However, these frugivorous lemurs are also excellent seed dispersers of invasive guava, potentially facilitating the spread of this invasive species. Although invasive fruit tree species can form a valuable energy source for lemurs in disturbed forests, such species may suppress the regrowth of native fruit tree species and may prevent the forest from attaining its original floristic diversity (Lowry et al., 1997). For that reason, we suggest as an important management measure to monitor the spread of invasive tree species, such as guava, that can potentially outcompete endemic fruit trees.

We found *H. griseus* in both the less disturbed and one intensely disturbed site, but we could not discern higher encounter rates in disturbed compared to less disturbed sites, as hypothesised. Although this species shows dietary differences in the disturbed forests, it does not show signs of reduced health (Grassi, 2001). Bamboo lemur species exhibit great variation in reaction to disturbance (Wright et al., 2008). Much of the secondary regrowth in our disturbed sites is dominated by clumps of giant bamboo (*Cathariostachys madagascariensis*). This bamboo species makes up a large part of the diet of *H. aureus* and *P. simus* (Arrigo-Nelson and Wright, 2004; Tan, 1999), while *H. griseus* is less specialised on this bamboo species (Grassi, 2006; Tan, 1999). Similar to Herrera et al. (2011), we only found one cluster of two *P. simus* individuals in a disturbed site (TALA). In contrast, although Herrera et al. (2011) found that *H. aureus* was only present in this previously disturbed site, we encountered this species in a less disturbed site (VALO) as well. *Hapalemur aureus* is known to be resident at only a few specific locations in Ranomafana NP and although other researchers also observed this species in VALO, no resident groups have been located yet (S.E. Johnson, *personal communication*). We have rarely encountered *P. simus* and only in a disturbed site (TALA). A previous survey found that *P. simus* is patchily distributed throughout Ranomafana NP, has specific microhabitat preferences, and specialises on giant bamboo (Arrigo-Nelson and Wright, 2004). It is known that a large population of *H. griseus* is resident in one of the disturbed sites (TALA) (Grassi, 2006; Herrera et al., 2011), but we did not encounter the species here. This is an indication that our results should be interpreted with caution, as our sampling effort is low for the extremely cryptic bamboo lemur species that have a patchy distribution and occur in relatively low densities. Bamboo lemur presence and population density likely respond to habitat disturbances, as such disturbances lead to habitat quality changes, including available food species. Nonetheless, a much more thorough investigation is needed to draw such conclusions.

Our results should be interpreted with caution due to the high variability in encounter rates among the transects within sites. In addition, we surveyed along pre-existing trail systems rather than sampling randomised transects, which may have

biased our estimates of species encounter rates (Buckland et al., 2010). At some parts, trails follow certain topographic features that make them easier to traverse, such as rivers or mountain ridges. Furthermore, the trails in these research sites were originally established by previous researchers for the explicit purpose of following known groups of animals in the area. This impacts the generalisability of our results, as the trails may have biased sampling to areas where some species have been known to occur. As a result, the estimates are likely to be higher than would be expected if the habitat would have been sampled randomly.

CLUSTER SIZE

Counter to our prediction that lemur cluster sizes would be smaller in previously disturbed forests, we could not detect such patterns in this study. Similar to the findings of previous studies, cluster sizes of *E. rubriventer, E. rufifrons*, and *P. edwardsi* did not differ between disturbed and less disturbed sites (Arrigo-Nelson, 2006; Tecot, 2008). Only *V. variegata* showed slightly larger cluster sizes ($P = 0.059$) in sites that experienced past anthropogenic disturbances, but this comparison should be viewed with caution, because sample sizes were small. Improved predator detection is an advantage of larger social groups, which has been shown in, for example, primates (Lehmann et al., 2007) and ungulates, like elk (*Cervus elaphus*) (Delm, 1990). Also competition for food resources is considered as a significant predictor of group size, with smaller groups being favoured when competition increases (Snaith and Chapman, 2007). Such feeding competition has shown to limit group size, for instance in lowland gorillas (*Gorilla gorilla gorilla*) (Parnell, 2002), and in many other primate and carnivore species (reviewed in Wrangham et al., 1993). The eastern rainforests of Madagascar show high habitat complexity and unpredictable food availability as a result of both human and natural disturbances, such as drought and cyclones (Ganzhorn, 1995; Ratsimbazafy, 2006; Wright, 1999). Small group sizes have been attributed to human disturbance and hunting (Parnell, 2002). Although a negative relationship between human presence and group size has been shown in other species, like bottlenose dolphins (*Tursiops truncatus*) (Constantine et al., 2004) and mountain gazelles (*Gazella gazella*) (Manor and Saltz, 2003), lemurs did not differ in group sizes in the disturbed areas that are more frequently visited by humans (i.e., eco-tourists, guides, and spotters). Overall, we found for all lemur species that cluster sizes were similar across forest sites that differed in disturbance intensity. This suggests that, by having relatively small cluster sizes, these lemurs have already adapted to living in heterogeneous and disturbed habitat.

CONCLUSIONS

Many forests worldwide have experienced anthropogenic forest exploitation, including selective logging, which influence forest structure and animal abundances (Gardner et al., 2007). Recovery and successional trajectories are site specific and depend on environmental conditions, land use histories, and management practices. Despite a recovery period of nearly 30 years, our results from a rainforest in Madagascar show that the impact of past logging can still be discerned in forest structural characteristics. Although the disturbed forests have not fully recovered to previous floristic conditions, they seem to have recovered from a functional perspective into suitable lemur habitat, as lemur encounter rates and cluster sizes were fairly similar across sites. Integrating structural and functional characteristics of regenerating forests is important in the successful management of forest ecosystems. Like many other lemur species in Madagascar, *Varecia variegata* underwent a drastic population decline due to decreased habitat quality and size. This is one of the first studies that recorded the reappearance of this large-bodied and obligate frugivore species in previously disturbed sites. Species such as *V. variegata* can facilitate the dispersion of large-seeded tree species, hereby promoting the recovery of sites where such trees were removed. The presence of seed-dispersing lemurs likely plays an important role in forests regeneration and in returning ecosystem function to pre-disturbance levels. Effective management of disturbed forests to achieve the recovery of ecosystem functions and the maximum potential for regeneration is needed. We emphasise the importance of conserving selectively-logged rainforest sites as the latter possesses considerable conservation potential for Madagascar's endangered lemur species, as well as overall biodiversity.

CHAPTER 3

THE COEXISTENCE OF TWO CONGENERIC LEMUR SPECIES

Niche separation and competition as underlying mechanism

de Winter, I.I., van Hooft, W.F., Koene, P., Larney, L, Heitkönig, I.M.A., Gort, G., Prins, H.H.T., Wright, P.C.

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ABSTRACT

Due to the relatively similar ecological characteristics of closely related species, there is still debate in community ecology about the causal mechanism behind their coexistence. Specifically, congeneric species are usually more alike in their biology, ecology, and morphology than more distantly related species and therefore, interspecific competition should constrain their coexistence. The coexistence of lemur species is relatively common in the genus *Eulemur* (true lemurs) compared to other lemur genera and primates in general. We question what mechanisms enable the coexistence of *Eulemur rufifrons* and *E. rubriventer*. Specifically, we test whether landscape heterogeneity, caused by logging, as well as niche differentiation and agonistic interactions facilitate their coexistence. To this end, we measured species encounter rates in five different sites within a rainforest in south-east Madagascar that experienced different levels of selective logging in the past. In one of these sites, we used a combination of focal continuous and instantaneous recording of the niche use of three social groups of *E. rufifrons* and four social groups of *E. rubriventer* and their direct intra- and interspecific interactions. We found significant differences in the encounter rates of the two species in different sites, indicating spatial separation between species. We also found differences between the species in their use of feeding trees as well as food items, positions in trees, and activities over the day. *Eulemur rufifrons* was more involved in intraspecific interactions than *E. rubriventer*. We propose that large-scale spatial segregation into different areas within a heterogeneous environment, caused by previous logging, in combination with niche differentiation, facilitates the coexistence of these congeneric species.

INTRODUCTION

The mechanisms for the coexistence of species within ecological communities remain one of the major topics in community ecology (Dammhahn and Goodman, 2014) and has long represented a fundamental question (Stokstad, 2009). There are limits to the similarity between co-existing species (Chase and Leibold, 2003), which makes the coexistence of congeneric species, i.e., species belonging to the same genus, difficult to explain. Due to their recent common ancestry, congeners can be quite equivalent: trophically, morphologically, and functionally, when compared to more distantly related species (Sfenthourakis et al., 2005). As a result, many similarities within the fundamental niches of congeneric species may be present (Schoener, 1982; Sinclair et al., 2006). However, niche separation seems to be inevitable, especially when food availability is limited, and facilitates the coexistence of such closely related species (Futuyma, 2013). This should affect congeners more than more distantly related species within a community, leading to increased species divergence (Chase and Leibold, 2003; Schoener, 1974).

It is likely that both niche differentiation and neutrality play a role in the coexistence of closely related species (Leibold and McPeek, 2006). From a neutral perspective, wherein species are assembled without respect to functional niches, equivalence of species can occur in communities as well (Leibold and McPeek, 2006). Species can be equivalent in most aspects of their population and evolutionary dynamics and interact with rest of the community as essentially one functional group (Leibold and McPeek, 2006; Urban et al., 2008). Biotic and abiotic stressors, as well as high species richness in a community strongly reduce the potential for competitive exclusion of functionally equivalent or nearly equivalent species (Hubbell, 2006). Thus, also from a neutral perspective, coexistence of similar species is sometimes favoured (Leibold and McPeek, 2006) and it is likely that both niche differentiation and neutrality play a role in the coexistence of species.

Although the coexistence of congeners remains debated (Chase and Leibold, 2003; Chesson, 2000; Dammhahn and Goodman, 2014; Prins and Gordon, 2014; Sfenthourakis et al., 2005), several mechanisms are known to promote their coexistence. First, spatial environmental heterogeneity can influence competition and coexistence patterns, which has been mathematically shown in multiple studies (reviewed in López-Gómez and Molina-Meyer 2006). For example, it is demonstrated that spatial heterogeneity can lead to higher species diversity (Neuhauser, 2001) and that patches of different disturbance histories within an area can enable the coexistence of species (Roxburgh et al., 2004). In addition to anthropogenic disturbances, including selective logging (de Winter et al., 2018a; Questad and Foster, 2008), spatial heterogeneity of forests can result from natural disturbances (van der Maarel, 1993). Although heterogeneity caused by disturbance seems to play an

important role in promoting coexistence in ecological communities (Dial and Roughgarden, 1998), it is not well understood yet (López-Gómez and Molina-Meyer, 2006).

Second, classic ecological niche theory provides a useful account of how competition can explain the coexistence of species (Chase and Leibold, 2003). Niche differentiation through coevolutionary processes along major niche dimensions, i.e., food resources, space, and time, is considered to lower competition between species (Chase and Leibold, 2003). Species can coexist by depressing their own population growth rates more than they depress the other species (Chesson, 2000). However, the relation between competition and niche separation or niche overlap is complex. In the traditional interpretation, a small dietary overlap between species indicates that the differences between two species in resource partitioning evolved by relatively intense interspecific competition. In contrast, high levels of resource overlap indicate shared resource use due to a lack of competition (Gotelli and Graves, 1996), but could also imply that competition is present, but has not yet led to divergence in resource use (e.g., Belovsky 1986; Jenkins and Wright 1988). Temporal variation, for example in food availability throughout a year, can also play a role in the competition between species (Questad and Foster, 2008). Especially when resources are limited, species are assumed to be forced into different niches and differential resource use becomes more apparent (Grøtan et al., 2012; Levin, 1970). In addition to trophic differences, species may show small-scale spatial differences to lower direct competition, for example in their feeding and resting locations (Hopkins, 2013). Species can also show differences in their daily activity pattern, as by being active at different parts of the day, they can lower direct feeding competition (Sussman, 1974; Vasey, 2006). Evaluating multiple aspects of niche separation is needed to explain the coexistence of species within communities (Amarasekare, 2003; Pianka, 1973; Sauther, 1993; Schoener, 1974).

Third, substantial niche overlap can induce direct interspecific competitive interactions between species, often including territorial behaviour like aggression and scent marking. When species engage in dominance interactions, the dominant species usually excludes the subordinate species from the preferred habitat (Heymann, 2003; Houle et al., 2006) and eventually, such dominant competitors may drive inferior competitors to extinction (Goreaud et al., 2002). Direct competitive interactions can also result in patchy distribution patterns, which supports the coexistence of functionally similar species in ecosystems (Diamond, 1975; Segura et al., 2013).

Despite these theories, there is still considerable confusion about the roles of disturbance, niche separation, and direct competition in promoting the coexistence of closely related species in ecological communities (Stokstad, 2009). Because the

mechanisms that can explain coexistence patterns often differ between ecological communities, determinants of these patterns need to be specifically examined for a given community or taxonomic assemblage (Schäffler et al., 2015).

In primate species, the geographic coexistence of congeneric species is rare (Houle, 1997), but it is more common in lemurs (Kamilar et al., 2014). Especially within the true lemur genus (*Eulemur*), coexistence of congeneric species is relatively common compared to other lemur genera, but the mechanisms enabling the patterns are not clear (Dammhahn and Goodman, 2014; Dammhahn and Kappeler, 2008; Rakotondravony and Radespiel, 2009). Therefore, our aim is to document the mechanisms enabling the stable coexistence of two lemur species: the red-fronted brown lemur (*Eulemur rufifrons*) and the red-bellied lemur (*E. rubriventer*). These *Eulemur* species live sympatrically in an eastern rainforest in Madagascar, Ranomafana National Park (NP), share many ecological characteristics, and are therefore likely to compete strongly with each other for access to critical food resources (Schoener, 1974). Other studies indeed found oscillating population densities of both species, which may reflect some degree of congeneric competition (Erhart and Overdorff, 2008; Johnson and Overdorff, 1999). Multiple ecological and behavioural studies have been performed on these species (e.g., Erhart and Overdorff 2008; Overdorff 1993; Overdorff 1996; Overdorff et al. 1998; Overdorff and Tecot 2006; Tecot 2008; Wright et al. 2012). Nevertheless, comparative studies on the large-scale spatial separation in areas that experienced differences in habitat disturbance, niche differences, and direct interactions in sympatric *Eulemur* populations have been lacking so far. In addition, most studies on competition and niche separation were performed in the dry season, which is usually a period of food scarcity (Wright et al., 2005), and only few documented lemur behaviour year round (Overdorff, 1993).

The sympatric, congeneric, and ecologically similar lemur species *E. rufifrons* and *E. rubriventer* in Ranomafana NP in the eastern Malagasy rainforest serve as a suitable model to examine the possible mechanisms underlying the coexistence of congeners. We question what mechanisms enable the coexistence of these species, and specifically, we test whether landscape heterogeneity caused by logging, as well as niche differentiation and agonistic interactions, facilitate their coexistence. We predict that (1) encounter rates of *E. rufifrons* and *E. rubriventer* are negatively correlated across sites with different disturbance histories; (2) the *Eulemur* species show trophic, spatial, or temporal niche differentiation; and (3) signs of direct competition, measured as agonistic interactions, between the two species are present. By recording encounter rates in five different forest sites in Ranomafana NP, we evaluate the large-scale spatial segregation between *E. rufifrons* and *E. rubriventer*. Within one of these sites, we used a combination of focal continuous and instantaneous recording of their niche use and direct inter- and intraspecific interactions.

METHODS

STUDY SITE

Ranomafana NP is located in southeastern Madagascar at 47°20'E and -21°16'S and consists of 43,500 hectares continuous rainforest (Wright and Andriamihaja, 2002). The park is home to at least twelve species of lemurs, including seven diurnal or cathemeral lemur species: *Hapalemur aureus, H. griseus, Prolemur simus, Propithecus edwardsi, Eulemur rufifrons, E. rubriventer*, and *Varecia variegata* and five nocturnal species: *Avahi laniger, Microcebus rufus, Daubentonia madagascariensis, Cheirogaleus major*, and *Lepilemur microdon* (Houston, 2017; Wright et al., 2012; Wright and Andriamihaja, 2002). In the wet season from December through March, temperatures range between 17 and 28°C and average rainfall is 400 mm per month. Although food availability in Ranomafana NP highly fluctuates within and between years, these months are considered as the period of high fruit availability, with fruits being the major food source for *Eulemur* species (Tecot, 2008; Wright et al., 2005). We performed this study in five different sites within Ranomafana NP: Talatakely (TALA), Sakaroa (SAKA), Vohiparara (VOHI), Vatoharanana (VATO), and Valohoaka (VALO). Previous disturbances, i.e., different intensities of selective logging, have created environmental heterogeneity within the forest (Balko and Underwood, 2005; de Winter et al., 2018a; Herrera et al., 2011; Wright et al., 1997). In TALA, SAKA, and VOHI, extensive clearing for agriculture and human habitation in the 1950s was followed by intensive commercial logging until the late 1980s. VATO and VALO are less disturbed sites, where commercial logging occurred with much lower intensity (Fig. 2.1). One of these sites, TALA, contains sufficiently large populations of *E. rufifrons* and *E. rubriventer* (Houston, 2017), and is therefore chosen for the behavioural part of this study.

STUDY SPECIES

In this study, we focus on two true lemur species (genus *Eulemur*, family Lemuridae): *Eulemur rufifrons* (red-fronted brown lemur) and *E. rubriventer* (red-bellied lemur). These species are medium-sized (body and tail length 30 to 50 cm) arboreal primates that occasionally move quadrupedally. They are morphologically alike with a body mass of 1.6 to 2.4 kg for *E. rubriventer* and 2.2 to 2.3 kg for *E. rufifrons* (Glander et al., 1992; Mittermeier et al., 2006). Their diet primarily consists of fruits (70%), flowers, and leaves (Erhart and Overdorff, 2008; Markolf et al., 2013; Overdorff, 1993, 1996; Overdorff et al., 1998; Overdorff and Tecot, 2006; Sato et al., 2016; Tecot, 2008; Wright et al., 2012). *Eulemur rufifrons* lives in multi-male, multi-female groups from four to 18 individuals (Overdorff, 1996) within relatively large home-ranges of about 100 ha (Andriaholinirina et al., 2014; Overdorff, 1993). *Eulemur rubriventer* lives in small monogamous groups from two to five individuals and has strict territories, with

relatively smaller home-ranges of 12 to 15 ha that are frequently defended by territorial behaviour, like scent-marking (Tecot, 2008, 2010; Tecot et al., 2016).

LEMUR SURVEY PROCEDURE

To assess the encounter rates of both lemur species in the different research sites, we conducted transect surveys between November 2014 and February 2015. For all the lemur measurements, we walked two-km long transect survey routes that followed a pre-existing trail system wherever possible to minimise forest impacts and to allow for more extensive surveys (Herrera et al., 2011; Hiby and Krishna, 2001; Lehman et al., 2006; Wright and Andriamihaja, 2002). We used curved line transects that we call 'transect surveys' and define a transect as a path along which one counts and records occurrences of the species of interest (Hiby and Krishna, 2001). The trail system in our sites was originally established for the explicit purpose of following known groups of animals. We walked the survey transects in a team of one observer and one assistant, trained at the Centre ValBio research station, at a slow, constant pace of one km/h. For each visual lemur encounter, we recorded the species and social cluster size. We consider our encounters to be with clusters of lemurs, instead of complete social groups, as we cannot assume that the entire social group is encountered at once (Plumptre and Cox, 2006). Large group spreads in general or the presence of individuals at the periphery of the observed cluster may elude detection, which would lead to an underestimation of the actual group size. Individuals of a species found < 50 m of each other along a transect survey were considered to be in the same cluster. As a proxy for abundance, we calculated lemur encounter rates as the number of clusters and individual lemurs per kilometre (Herrera et al., 2011). We conducted three non-overlapping transect surveys per site (total: fifteen different survey routes) that we repeatedly (four to ten times) surveyed. We alternated the time and transect survey in a way that transect surveys were not measured more than once every 18 hours (Herrera et al., 2011). Each day, we walked three transect surveys at different times of the day: < 09:00 h, 09:00-12:00 h, and > 12:00 h. When weather conditions reduced the visibility to less than 15 meters, no survey was conducted. This transect survey method is well established and is suitable for the arboreal primate species we study (Herrera et al., 2011; Irwin et al., 2005; Johnson and Overdorff, 1999; Lehman, 2007). Based on a total search effort of 210 km, this led to 36 actual lemur cluster encounters across the two species at five sites, three different transects surveys per site that we surveyed four to ten times, yielding 19 to 23 transect surveys per site with a total of 105 surveys.

BEHAVIOURAL DATA

From December 2010 through February 2011, the wet season, we performed one-day follows on one adult male and one adult female within the same social group

simultaneously with four observers, two observers per focal animal, between 07:00 h and 17:30 h, five to six days a week. We excluded all data before 9:00 h and after 16:00 h, as in these timeframe, the number of observations was low for one of the species, leading to an average observation length of 6.3 hours a day. We alternated species and social groups daily, so that resource availability and weather conditions would be as similar as possible for both species (Altmann, 1974). We recognised focal individuals by characteristics like sex, body size, and potential scars or collars that were left from other behavioural studies.

By documenting food preferences, spatial preferences, and temporal activity patterns of sympatric social groups of *E. rufifrons* and *E. rubriventer*, we explored the niche separation between these two species. For each focal animal, we recorded all occurrences of feeding (continuous recording), including the duration of a feeding bout, the food tree species, and the consumed food items. Food items were categorised in: ripe fruits, unripe fruits, leaves, flowers, and other items, e.g., fungi, bark, and soil. Furthermore, we used focal instantaneous recording of behaviours in five-minute intervals according to predefined categories: feeding, grooming, playing, resting, travelling, and other/out of sight (Overdorff, 1993; Vasey, 2006). The location of the lemur species in a tree was recorded as follows: on the ground (ground), on the trunk (trunk), in the lower canopy close to the trunk (LC1), in the lower canopy in the terminal branches (LC2), in the upper canopy close to the trunk (UC1), and in the upper canopy in the terminal branches (UC2). In total we observed 66 individuals in seven social groups (132 day follows, totalling 414.5 hours and 4974 five-min records): 26 different adult individuals of *E. rubriventer* (13 males, 13 females) in four different groups (52 day follows, totalling 173.4 hours and 2081 five-min records) and 40 adult individuals of *E. rufifrons* (20 males, 20 females) in three social groups (80 day follows totalling 241.1 hours and 2893 five-min records).

For both *Eulemur* species, we described all intraspecific interactions (i.e., with conspecific groups) and interspecific interactions (i.e., between groups of the different lemur species). Also, interactions with other lemur species within the diurnal lemur community within Ranomafana NP were described. To examine the intraspecific and interspecific interaction rates and the intensity of direct competition between the two species, we took *ad libitum* notes on potential vocalisation and the expression of dominance behaviour (Altmann, 1974). We distinguished two categories of interactions: (1) displacement of a group with potential vocalisations; and (2) a fight that includes physical contact, biting, scratching, coughing, and/or chasing. We also noted the winning and losing group, which is, respectively, the group that succeeded to stay in the area and the group that was chased away (Altmann, 1974).

All the non-invasive research included in this chapter was performed within Ranomafana NP and has been in compliance with the laws of the government of

Madagascar. The research was approved by the trilateral commission (CAFF/CORE) in Madagascar (permits 297/13 and 143/14/MEF/SG/DGF/DCB.SAP/SCBSE).

STATISTICAL ANALYSIS

For the lemur encounter rates, we first calculated the mean number of cluster and individual encounters per kilometre for each species within the five research sites. Next, we used Spearman's rank correlation to examine correlations between the mean encounter rates of the two species, both for the cluster and individual encounters. Per species, we compared group sizes between the five sites using a generalised linear mixed model (GLMM) with Poisson distribution and log link (Bolker et al., 2009), using species, site, and their interaction as fixed effects; transects and surveys within transects as random effects; and the logarithm of the number of clusters as offset. Statistical analyses were performed using R (version 3.4.1, R CoreTeam, 2017) and R-package lme4 (version 1.1-1.3, Bates et al., 2015), with add-on packages for testing and prediction (lmerTest version 2.0-33, pbkr version 0.4-7; lsmeans version 2.27-2).

We examined niche separation by studying species specific tree preferences, the use of feeding items, diurnal activity patterns, locations within trees, and intra- and interspecific interactions. To describe and compare the lemurs' preferences for specific food tree species, we calculated the percentage of time the species spent feeding in a tree species. Next, to compare the two *Eulemur* species in the number of tree species that an individual lemur used for feeding, we fitted a GLMM (with Poisson distribution and log link) to the number of tree species an individual visited. Here, we corrected for observation duration, percentage of observation time during the species' active period, and sex (using fixed effects) and for day of observation and social group (using random effects). To compare the two species, we calculated predicted means with 95% confidence intervals (CI) at average covariate values.

The index for dietary overlap was calculated using Pianka's index (Pianka, 1973). To assess whether the probability that the observed dietary overlap values between the two species were more overdispersed than expected by chance, we compared the observed data matrix with randomised pseudo-communities generated by 1000 Monte Carlo simulations in null model tests using EcoSim V. 7.72 (Gotelli and Entsminger, 2006; Pianka, 1986). We used 'Randomised Algorithm 3' to calculate expected niche overlap indices, as this algorithm retains niche breadth, and thus the amount of specialisation on specific resources, during the randomisation process (Winemiller and Pianka, 1990).

We analysed the fraction of observations for a specific feeding item, activity, and position in the tree with GLMMs using a binomial distribution and logit link. We included fixed effects for sex, species, part of the day, and the interaction between species and part of the day, and random effects for social group, date, and the lemur individual. Part of the day was defined as a factor with three levels: morning= 9:00-

10:59 h, mid-day= 11:00-13:59 h, and afternoon= 14:00-15:59 h. Due to the low number of feeding bouts on specific food items or behavioural recordings, we excluded the food items 'flowers' and 'other items' as well as 'other behaviour/out of sight' from our analyses. For locations in the tree, we also tested whether the species differ in their use of exposed locations. Therefore, we grouped the more exposed positions: ground, LC2, and UC2 and the less exposed positions: trunk, LC1, and UC1.

We analysed interspecific interactions between *E. rufifrons* and *E. rubriventer* with a binomial test, to examine whether one of the two species was dominant over the other. For intraspecific interactions we refrained from a hypothesis testing approach because of sparsity of observations, i.e., only a single intraspecific interaction for *E. rubriventer* was observed.

RESULTS

SPATIAL SEGREGATION

We found a negative correlation between the encounter rates of both clusters and individuals of *E. rufifrons* and *E. rubriventer* (both Spearman rank correlations: ρ = -1, P = 0.017). *Eulemur rubriventer* showed relatively high and *E. rufifrons* relatively low encounter rates in VALO and VATO (i.e., less disturbed sites) and in VOHI, while the opposite pattern holds for SAKA and TALA (i.e., highly disturbed sites) (Fig. 3.1). The group sizes of both species were not significantly different between sites (*E. rufifrons*, P = 0.36, *E. rubriventer*, P = 1.00, Table 3.1).

Table 3.1: Social group sizes of *Eulemur rufifrons* and *E. rubriventer*. Group sizes are given for five sites within Ranomafana National Park: Talatakely (TALA), Sakaroa (SAKA), and Vohiparara (VOHI) (i.e., with high disturbance intensity), and Vatoharanana (VATO), and Valoahaka (VALO) (i.e., with low disturbance intensity).

	More disturbed			Less disturbed		
	SAKA	**TALA**	**VOHI**	**VALO**	**VATO**	**Average**
Eulemur rufifrons	9.27	7.17	8.00	5.50	7.50	7.71
Eulemur rubriventer	3.50	3.00	3.60	3.25	3.00	3.29

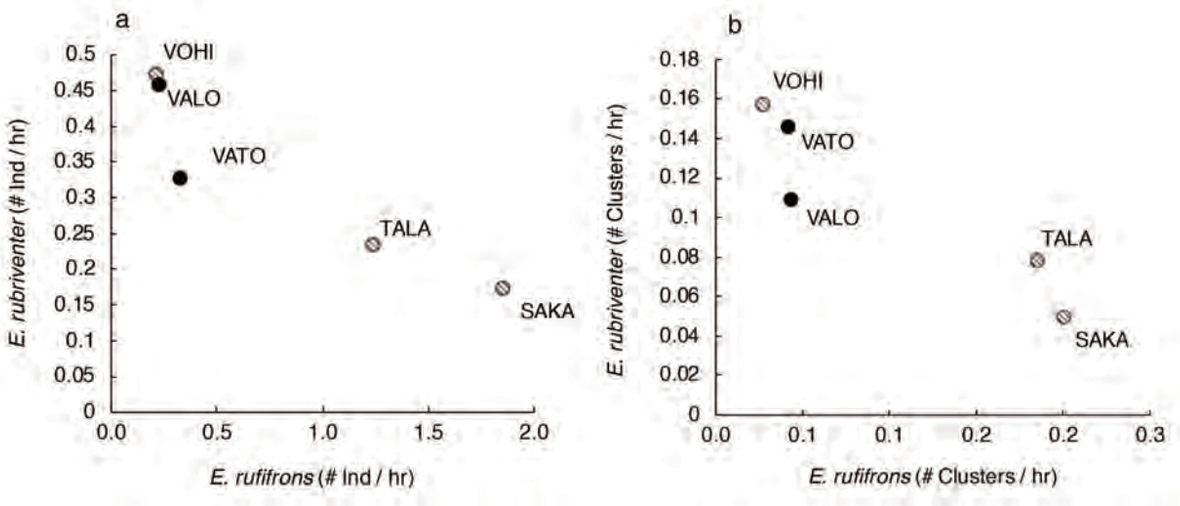

Figure 3.1: Lemur encounter rates. Encounter rates per kilometre of a) individuals and b) clusters of *Eulemur rubriventer* (y-axis) and *E. rufifrons* (x-axis) at five sites within Ranomafana National Park: Talatakely (TALA), Sakaroa (SAKA), Vohiparara (VOHI) (i.e., with high disturbance intensity, striped dots) and Vatoharanana (VATO) and Valohoaka (VALO) (i.e., with low disturbance intensity, solid black dots).

RESOURCE USE

Eulemur rufifrons used 4.16 (CI 3.32 - 5.21) and *E. rubriventer* used 3.2 (CI 2.46 - 4.26) different tree species per average observation (duration 6.3 hours), and these numbers were not significantly different between species (GLMM, $Z = 1.38$, $P = 0.17$). Both ripe and unripe fruits of the tree species *Aphloia theiformis* (Fandramanana), *Scolopia* sp. (Faritraty), and *Streblus dimepate* (Mahanoro) formed more than 40% of both species' diet, but the specific importance of each tree species seemed to differ between the two lemur species (Fig. 3.2). For example, *E. rubriventer* included a higher proportion of *Scolopia* sp. in its diet, while *E. rufifrons'* diet had a higher proportion of *Streblus dimepate*. Additionally, each lemur species foraged on some unique tree species: *Eulemur rufifrons* was the only species feeding on *Grewia humblotii* (Hafipotsy), while *E. rubriventer* was the only species feeding on *Oncostemum botryoides* (Kalafana) and *Trichodypsis hildebrandtii* (Tsirika). We listed all tree species, as well as soil and fungi, used by both species during our complete observation period (supplementary material, Table 3.4). The observed niche overlap (Pianka's index) in terms of feeding trees was 0.41 and did not significantly differ from the expected overlap simulated by a neutral model (Expected value = 0.44, P (Obs. < Exp.) = 0.45).

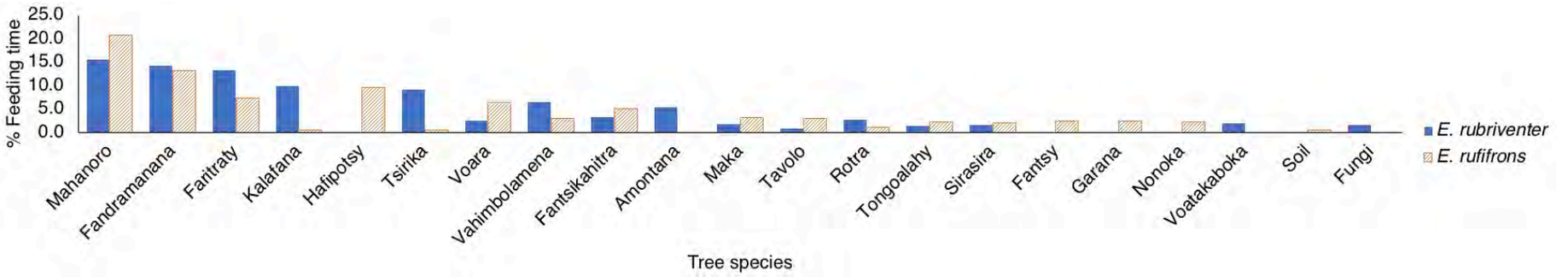

Figure 3.2: Use of feeding tree species. Percentage of feeding time spent on specific tree species (Malagasy nomenclature), soil, and fungi that represent more than 2% of the diet of *Eulemur rubriventer* (blue, solid) and *E. rufifrons* (orange, striped).

With regard to feeding on ripe fruits, unripe fruits, and leaves, the two species did not differ in the relative frequencies of these food items over the three parts of the day (ripe fruits: likelihood-Ratio Test *(LRT)* = 0.37, *P* = 0.83; unripe fruits: *LRT* = 1.91, *P* = 0.38; and leaves: *LRT* = 3.99, *P* = 0.14, Fig. 3.3). In addition, no differences in relative feeding frequencies of these food items were found between species (ripe fruits: *LRT* = 1.81, P = 0.18; unripe fruits: *LRT* = 0.43, *P* = 0.51; and leaves: *LRT* = 3.28, *P* = 0.070). Furthermore, we found no differences in relative feeding frequencies between males and females (ripe fruits: *LRT* = 0.17, *P* = 0.68; unripe fruits: *LRT* = 0.10, *P* = 0.75; and leaves: *LRT* = 0.00, *P* = 0.98). However, feeding patterns were significantly different between the three parts of the day (ripe fruits: *LRT* = 6.39, *P* = 0.041; unripe fruits: *LRT* = 7.24, *P* = 0.027; and leaves: *LRT* = 6.39, *P* = 0.041). Compared to the morning and mid-day, both species spent more time feeding leaves and seem to spend less time feeding on ripe fruits in the afternoon (leaves: *Z* = 2.31, *P* = 0.021; ripe fruits: *Z* = -2.65, *P* = 0.056), while they spent more time feeding on unripe fruits during mid-day and the afternoon when compared to the morning (mid-day: *Z* = 2.31, *P* = 0.021; afternoon: *Z* = 2.08, *P* = 0.038).

LOCATION IN TREES

With regard to the position in trees, the two species did not differ in the relative frequencies of locations in trees (*LRT* = 1.1 *P* = 0.2942, Fig. 3.4). The two species showed differences in the use of exposed positions within a tree at different times of the day (interaction position x part of day: *LRT* = 77.4, *P* < 0.0001). During the morning and afternoon, the two species showed comparable relative frequencies of time at exposed positions of the tree (morning: *Z* = -0.49, *P* = 0.62; afternoon: *Z* = 0.27, *P* = 0.50), but at mid-day *E. rufifrons* showed a remarkable preference for exposed positions compared to *E. rubriventer* (*Z* = -2.82, *P* = 0.0047).

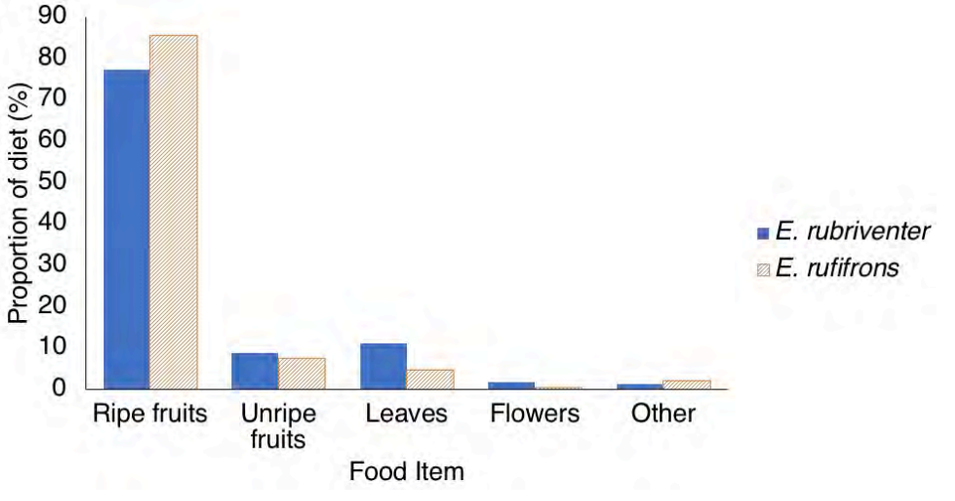

Figure 3.3: Food items. The proportion of time a specific food item was consumed by *Eulemur rubriventer* and *E. rufifrons*.

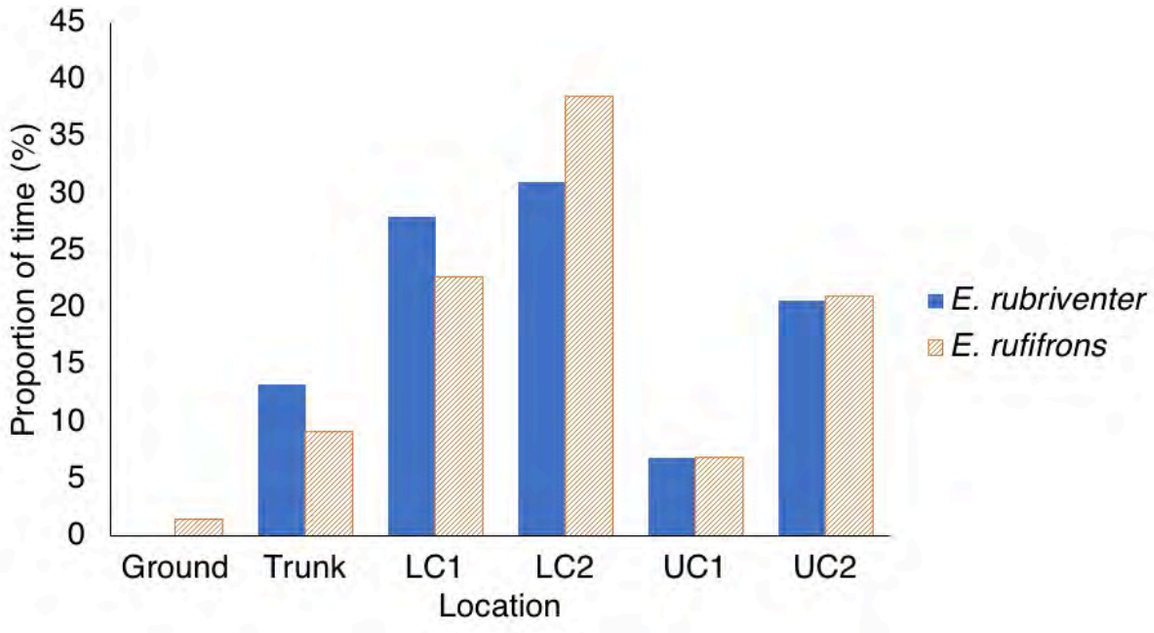

Figure 3.4: Proportion of time *Eulemur rubriventer* and *E. rufifrons* spent on different positions. Abbreviations: LC1: in the lower canopy, close to the trunk; LC2: in the lower canopy, in the terminal branches; UC1: in the upper canopy, close to the trunk; and UC2: in the upper canopy, in the terminal branches.

DIURNAL TIME-BUDGET

Both species spent most time resting and spent the same fraction of time on feeding and grooming (Fig. 3.5). In general, *Eulemur rubriventer* rested significantly more than *E. rufifrons* ($Z = 2.3$, $P = 0.02$), while *E. rufifrons* played ($Z = 3.83$, $P < 0.001$) and travelled relatively more than *E. rubriventer* ($Z = 2.96$, $P = 0.003$). When considering the part of the day, the species differed significantly in the time they exhibited specific behaviours (Table 3.2, Fig. 3.6). *Eulemur rufifrons* spent more time feeding and travelling in the afternoon when compared to *E. rubriventer*, while *E. rubriventer* spent more time resting in the afternoon when compared to *E. rufifrons*.

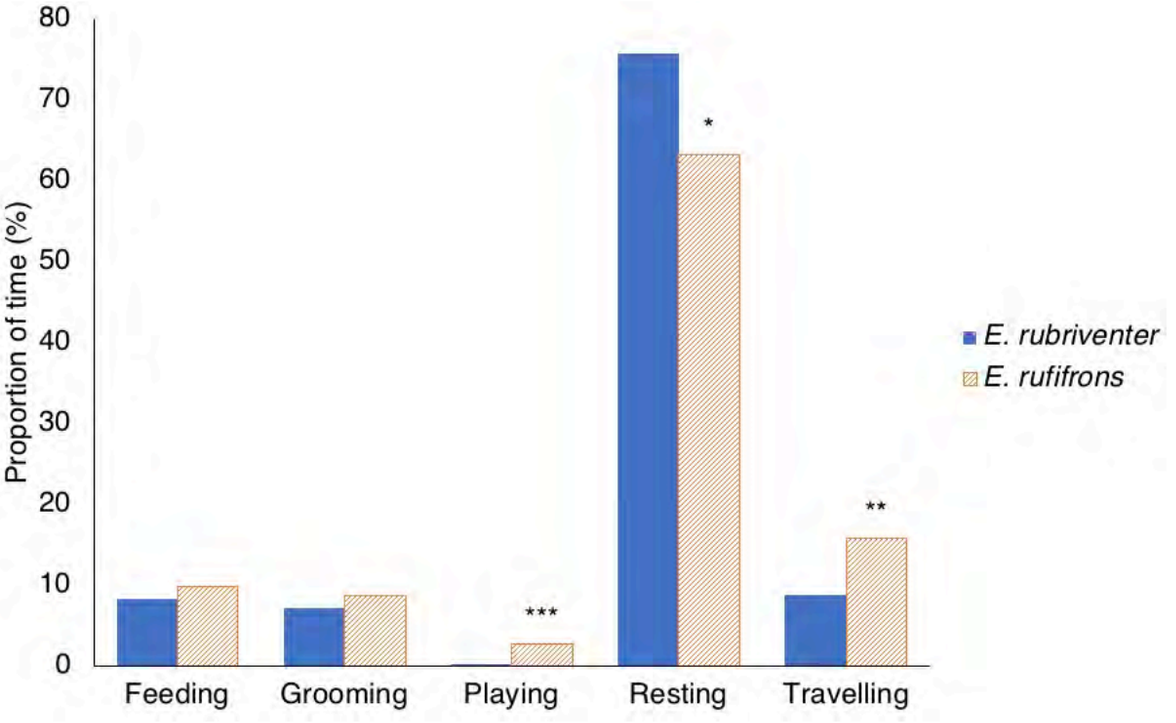

Figure 3.5: Proportion of time *Eulemur rubriventer* and *E. rufifrons* spent on different activities. Significant differences of the GLMM, $P < 0.05$ are marked with *, $P < 0.01$ with **, and $P < 0.001$) with ***.

Table 3.2: Overview of the different behaviours at different parts of the day. We give the estimated relative frequencies of each behaviour for *Eulemur rubriventer* and *E. rufifrons* during different parts of the day and compare these frequencies between these species.

Activity	Part of the day	Est. rel. freq. *E. rub.* – *E. ruf.*	*Z*-value	*P*-value
Feeding	Morning	0.111 - 0.107	0.20	0.84
	Mid-day	0.073 - 0.067	0.41	0.052
	Afternoon	0.057 - 0.127	-3.66	**<0.0001**
Grooming	Morning	0.105 - 0.100	0.17	0.86
	Mid-day	0.044 - 0.052	-0.53	0.60
	Afternoon	0.063 - 0.104	-1.58	0.11
Playing	Morning	0.0019 - 0.012	-1.80	0.072
	Mid-day	0.0005 - 0.0024	-1.08	0.28
	Afternoon	0.0000 - 0.040	-0.01	0.99
Travelling	Morning	0.113 - 0.169	-1.68	0.09
	Mid-day	0.074 - 0.074	0.00	0.99
	Afternoon	0.052 - 0.232	-5.73	**<0.0001**
Resting	Morning	0.641 - 0.580	0.82	0.41
	Mid-day	0.801 - 0.792	0.16	0.87
	Afternoon	0.819 - 0.443	5.52	**<0.0001**

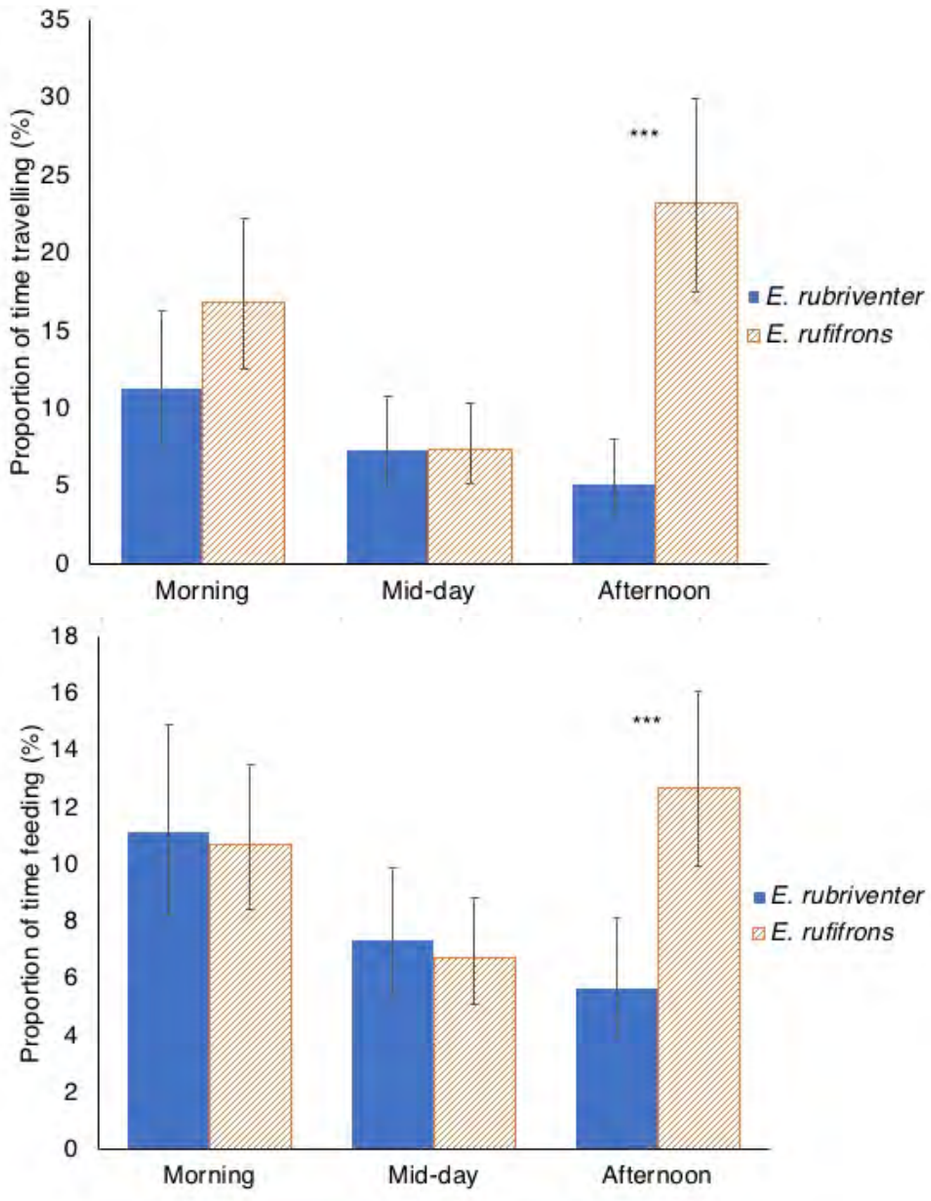

Figure 3.6: Proportion of time *Eulemur rubriventer* and *E. rufifrons* spent travelling (above) and feeding (below). Significant differences of the GLMM, $P < 0.05$ are marked with *, $P < 0.01$ with **, and $P < 0.001$) with ***. Error bars represent 95% confidence intervals.

SPECIES INTERACTIONS

During the 414.5 hours of observations, we observed nine intraspecific interactions in total: one between two conspecific groups of *E. rubriventer* (interaction rate = 0.006 interactions/hour) and eight between groups of *E. rufifrons* (interaction rate = 0.033 interactions/hour). The single interaction between the two *E. rubriventer* groups did not involve any aggression, as one group replaced the other in a feeding area without physical contact but with vocalisations. From the eight encounters between *E. rufifrons* groups, three were recorded as fights that involved aggression, including

agonistic vocalisations, chasing, scratching, or biting, while the other five interactions were displacements that all involved vocalisations. All fights involved a lot of scent marking by both species.

In total, we recorded fourteen interspecific interactions between *E. rufifrons* and *E. rubriventer* (interaction rate = 0.034 interactions/hour, Table 3.3) that were all in close proximity to known feeding trees. *Eulemur rufifrons* seemed to have won more interactions than *E. rubriventer* (ten out of thirteen interactions), but only a near significant difference was detected due to the low number of definite fights (binomial test, $P = 0.092$). For one fight we could not detect a winning species, as both species moved away from each other in different directions.

We recorded a few other interactions between these *Eulemur* species and other lemur species in Ranomafana NP. We observed six encounters between *E. rufifrons* and the relatively large-bodied frugivore *Varecia variegata* (interaction rate = 0.025 interactions/hour). *Eulemur rufifrons* was submissive in all interactions and was consistently replaced by *V. variegata*. No physical contact was observed, but both species vocalised when they came near each other (< 5 m in two occasions). Furthermore, *E. rufifrons* once encountered two bamboo lemur species: one individual of *Hapalemur griseus* and one social group of *H. aureus*. Both interactions involved vocalisations from both groups and *E. rufifrons* displaced these species in both occasions. *Eulemur rubriventer* only interacted once with another diurnal lemur species: *Propithecus edwardsi*. This interaction involved some vocalisations but no physical contact and *P. edwardsi* displaced *E. rubriventer.*

Table 3.3: Overview of the intra- and interspecific interactions and winning species recorded for *Eulemur rubriventer* and *E. rufifrons*.

	Intraspecific		Interspecific # won		
Species	**Displacement**	**Fight**	**Displacement**	**Fight**	**Total**
E. rubriventer	1	0	3	0	3
E. rufifrons	5	3	7	3	10
Total	**6**	**3**	**10**	**3**	**13**

DISCUSSION

There are still considerable discussions about the role of environmental heterogeneity, niche separation, and competition in structuring communities (Prins and Gordon, 2014; Stokstad, 2009). We aimed to contribute by investigating the coexistence of congeneric lemur species in an eastern rainforest in Madagascar. First, by recording species encounter rates in five heterogeneous sites in Ranomafana NP that experienced different logging intensities in the past, we obtained results on the spatial segregation between *E. rufifrons* and *E. rubriventer*. Second by using a combination of focal continuous and instantaneous recordings of the lemur's resource use, diurnal activities, and spatial positions in trees, we acquired results on potential niche differences between these two species. Third, by recording direct species encounters, we were able to evaluate the potential competition present within these diurnal lemur species in the community. Here, we discuss what factors promote the coexistence of our two study species and how our results contribute to understanding the coexistence of congeneric lemur species in general.

SPATIAL SEGREGATION

Differentiation among species in exploiting environmental heterogeneity can facilitate coexistence (López-Gómez and Molina-Meyer, 2006). The encounter rates of the congeneric species *E. rufifrons* and *E. rubriventer* were negatively correlated: *Eulemur rubriventer* showed relatively high and *E. rufifrons* relatively low encounter rates in two of the less disturbed sites, VALO and VATO, and in VOHI, while the opposite pattern holds for the disturbed sites SAKA and TALA. The different disturbance histories of these sites have created environmental variability, as the sites differ in multiple forest characteristics as well as tree species composition (de Winter et al., 2018a). The lemur species showed no clear preference for sites that experienced high or low disturbance intensities. Although *E. rufifrons* was more encountered in two more intensely disturbed sites and *E. rubriventer* in two less disturbed sites, *E. rubriventer* showed higher encounter rates in another disturbed site as well. This confirms that these lemurs can use both more and less disturbed sites as habitat (de Winter et al., 2018a) and that anthropogenic disturbance allows co-existence of these species. Our results are in line with other studies that suggest that variation in habitat characteristics selects for spatial segregation, which reduces interspecific competition and allows the coexistence of species (e.g., Myers et al. 2000; da Fonseca and Robinson 1990; Neuhauser 2001; Roxburgh et al. 2004, Dial and Roughgarden 1998; López-Gómez and Molina-Meyer 2006).

RESOURCE USE

Niche separation in resource use forms another postulated coexistence mechanism. In contrast to our prediction, our results demonstrate considerable trophic overlap between *E. rufifrons* and *E. rubriventer*. The species were rather similar in the number of tree species they used for feeding, but each species used some unique tree species. Their diet overlap in terms of the use of food tree species was 41%, which was not different from the expected overlap. This value corresponds with the dietary overlap that was observed in other studies on these *Eulemur* species (34% to 50% during peak fruit availability) (Overdorff 1993). In the wet season, when fieldwork was performed, fruit might not be a limiting resource for these frugivorous lemurs and both species indeed overlap to a certain extent in the tree species they use. This overlap is known to decrease to 6% during periods of fruit scarcity (Overdorff, 1993), which indicates that the species lower their resource overlap in periods of limited food supply to reduce competition (Grøtan et al., 2012; Levin, 1970). Thus, interspecific competition between the species may be present, but is not pronounced during periods of high food availabilities.

We observed that both *Eulemur* species focused their feeding on ripe fruits, but *E. rubriventer* included more leaves in its diet compared to *E. rufifrons* (Chase and Leibold, 2003; Schoener, 1974; Terborgh, 1985). Such subtle differences in the use of food items promote the co-existence of species (Pianka, 1973) and being flexible in the use of food items that are more difficult to digest, like leaves, can be advantageous in periods of food scarcity (Overdorff and Johnson, 2003). In these lemurs, gut capacity is limiting, like in other small mammals (Cork, 1996), and selecting high quality foods would be the optimal strategy to maximise the digestion rate and to acquire sufficient energy (Caton et al., 1996). Especially when ripe fruits are in short supply and one of the species turns out to be a stronger competitor, this species may replace the inferior species from high quality food patches. Switching to leaves or other food items can therefore be a coping mechanism for an inferior competitor, especially in periods of food scarcity.

Both species ate more ripe fruits in the morning and leaves and unripe fruits later in the day. Higher quality foods, such as ripe fruits are relatively rapidly digested. By eating such foods, animals obtain energy that they can directly use for their daily activities. In contrast, leaves and unripe fruits contain complex polysaccharides that require microbial fermentation and a longer retention time to obtain energy. Therefore, consuming lower quality foods before relatively long resting bouts during the night is a favourable digestion strategy that has also been observed in other primate species, like common marmosets (*Allithrix jacchus*) (Caton et al., 1996).

LOCATION IN TREES

In addition to these differences in resource use, we expected both species to show small-scale spatial differences in terms of their location in trees. It is possible that such differences could allow sympatry by reducing direct competition. The two species used certain positions in the tree at different times of the day. Although both species spent a lot of time at exposed positions within a tree in the morning and afternoon, *E. rufifrons* showed a remarkable preference for exposed positions during mid-day when compared to *E. rubriventer*. Furthermore, *E. rufifrons* was the only species that regularly came to the ground to play or feed on soil, mostly in the afternoon. Both *Eulemur* species may experience predation pressures from fossas (*Cryptoprocta ferox*), Madagascar harrier-hawks (*Polyboroides radiatus*), and Madagascar buzzards (*Buteo brachypterus*) (Karpanty and Wright, 2007; Wright et al., 2012). Being on the ground or at exposed positions may increase predation risks (Gautier-Hion et al., 1993; Overdorff, 1993). Larger social groups, like the groups of *E. rufifrons*, are known to benefit from their group size in their capacity to detect predators (Sussman and Garber, 2007; van Schaik, 1983), which may explain why this species takes more risks by being more exposed and by coming to the ground. We also noticed that *E. rufifrons* foraged relatively often in large fruit trees that bore many fruits on the outer branches (e.g., the fig species *Ficus lutea* and *F. tilifolia*), while *E. rubriventer* foraged more in relatively small tree species (e.g., *Scolopia* sp. and *Ludia* sp). Such a dichotomy has been shown to contribute to the co-existence of several other congeneric species (Noble et al., 2011; Pianka, 1973).

DIURNAL TIME-BUDGET

In our study, *E. rufifrons* spent more time playing and travelling than *E. rubriventer*, while *E. rubriventer* rested more. It is generally assumed that larger groups of primates, like *E. rufifrons*, use larger home ranges, exploit widely dispersed resources, and spend more time travelling in search for favourable food patches, compared to smaller groups, like *E. rubriventer* (Isbell, 1991; Janson and Goldsmith, 1995). We found no differences in daily feeding time between both species, so both species were not time-limited, which can be explained by the relatively high food abundance in this season. It has been suggested that large social groups focus on food patches that are large enough to support all group members simultaneously (Overdorff, 1993), while smaller groups can be more flexible in the size of food patches they choose (Schoener, 1971). So, the small group size of *E. rubriventer* may allow this species to be more flexible to resource density, which relaxes local resource competition (Stevens and Willig, 2000).

The largest behavioural differences were found in the afternoon, when *E. rufifrons* was more active than *E. rubriventer*. Lowering the species potential overlap in time by feeding and travelling at different times of day may be to both lemur species'

advantage, as it lowers the potential for direct interactions (Overdorff, 1996; Pianka, 1973). Due to these differences, species can be more limited by conspecific groups than by sympatric, interspecific groups, which can lead to stable local coexistence of two species (Amarasekare, 2003; Amarasekare et al., 2004; Chesson, 2000). We propose that such differences in diurnal time-budget play an important role in species coexistence in general and could therefore be the focus of future studies on this topic.

SPECIES INTERACTIONS

In general, congeneric species, with similarities in ecology, morphology, and behaviour, experience relatively intense interspecific competition when compared to species with a less recent ancestry (Sfenthourakis et al., 2005). We therefore expected the two *Eulemur* species to show strong competition. Indeed, compared to all other lemur species within Ranomafana NP, including some other frugivorous species like *Varecia variegata* and *Propithecus edwardsi*, the congeneric *Eulemur* species seem to compete most strongly with each other (Wright et al., 2012), likely due to their relatively recent common ancestry and functional similarities. This is in line with other studies that confirm higher rates of aggression in phylogenetically close primate species (Houle, 1997; Houle et al., 2006). Like another study, we observed that most encounters between the two species occurred during travel towards a food source or while groups were feeding (Overdorff and Tecot, 2006). As most agonistic interactions occurred in areas with ripe fruit (Johnson et al., 2005; Overdorff and Tecot, 2006), interference competition for fruit is the most likely driver of the aggressive attempts to exclude one another from feeding areas.

We found that social groups of *E. rufifrons* were more engaged in agonistic activities and the species interacted significantly more with conspecific groups compared to *E. rubriventer*, similar to what was shown in a previous study (Overdorff, 1996; Overdorff and Tecot, 2006). In interspecific interactions between the two species, *E. rubriventer* was either displaced or actively chased from a feeding area by *E. rufifrons*, suggesting that *E. rufifrons* displays feeding priority over *E. rubriventer*. This might be explained by the larger group sizes of *E. rufifrons* compared to *E. rubriventer*, as *E. rufifrons* lives in multi-male, multi-female groups of on average about nine individuals (de Winter et al., 2018a; Johnson and Overdorff, 1999; Kappeler and Fichtel, 2016; Overdorff et al., 1998), while *E. rubriventer* lives in pair-bonded family groups with on average three individuals (Overdorff, 1993, 1996; Tattersall and Sussman, 2016). Furthermore, the parent-offspring ratio was larger in groups of *E. rufifrons*, which enables combining resource defence and the protection of their infants. Other primate species with relatively large group sizes have been observed to displace sympatric pair-bonded species from fruit trees as well (Terborgh, 1985).

Overdorff & Tecot (2006) found that the majority of interactions (88%) between

the two species took place during periods with high food availability and rates of interspecific encounters were twice as high during high food availability (0.06/h) than during food scarcity (0.03/h). Overdorff and Tecots' study was done in one relatively undisturbed site (VATO), while we found an interaction rate of 0.034/h in a relatively more disturbed site (TALA). In areas that experienced human disturbances, lemur interaction rates might therefore be lower. Nevertheless, the direct interactions we recorded in this study, especially events where aggression was observed, form an indication that competition between the two species is still present (Connell, 1980; Sale, 1974).

We need to put forward that both *E. rubriventer* and *E. rufifrons* are known as cathemeral species, which means that they can be active throughout the 24-hours cycle (Overdorff and Rasmussen, 1995), so nighttime interactions were missed. Especially in sites that experience anthropogenic disturbance, *Eulemur* species seem to become less active during the day (Donati et al., 2016), as was observed earlier for *E. rubriventer* in disturbed sites (Tecot, 2008). Therefore, to fully understand activity patterns of both species, we suggest other researchers to include nocturnal activity patterns, to be able to evaluate the role of cathemeral behaviour in explaining coexistence patterns. Furthermore, we collected data during the wet season, while both species may exhibit different interaction rates and behaviour during the dry season (Curtis and Rasmussen, 2006). Collecting year-round data over several years would be needed for a more complete picture of the coexistence patterns of these species.

CONCLUSIONS

Many lemur communities in Malagasy forests include congeneric species and understanding their coexistence provides important ecological insights. Different disturbance histories have created spatial heterogeneity within the eastern rainforest in Madagascar of this study, which has led to large-scale spatial segregation of both species. This limits interspecific interference competition among congeneric lemur species and hence facilitates their coexistence. Lemurs radiated into more than one hundred different species, comprising more than 20 percent of all primate species that are known today. This study adds to the understanding of lemur coexistence and thereby to the extraordinary diversity in these primates. Extreme differences in ecosystems and habitats in Madagascar, in combination with environmental heterogeneity caused by anthropogenic disturbances, facilitates the coexistence of multiple lemur species. Based on encounter rates, a quantitative behavioural study on niche partitioning, and direct interactions in two *Eulemur* species, we propose that, in addition to niche differentiation (i.e., in resource use, time, and space), large-scale spatial segregation into different areas within a heterogeneous environment, caused by logging, is an important mechanism explaining the coexistence of these two congeneric lemur species and potentially other closely related species within primate communities.

SUPPLEMENTARY MATERIAL

Table 3.4: Use of feeding tree species. Percentage of feeding time spent on specific tree species (Malagasy nomenclature), soil, and fungi for *Eulemur rubriventer* and *E. rufifrons*.

Tree Species	Feeding time *E. rubriventer* (%)	Feeding time *E. rufifrons* (%)	Total (%)
Mahanoro	15.58	20.67	36.25
Fandramanana	14.25	13.13	27.38
Faritraty	13.30	7.41	20.71
Kalafana	9.82	0.56	10.38
Hafipotsy	0.00	9.52	9.52
Tsirika	9.04	0.46	9.50
Voara	2.54	6.39	8.93
Fantsikahitra	3.24	5.14	8.38
Amontana	5.35	0.00	5.35
Vahimbolamena	6.42	2.98	9.41
Maka	1.67	3.12	4.80
Tavolo	0.84	3.04	3.88
Rotra	2.63	1.15	3.77
Tongoalahy	1.35	2.29	3.64
Sirasira	1.57	2.07	3.64
Fantsy	0.00	2.38	2.38
Garana	0.00	2.34	2.34
Nonoka	0.00	2.20	2.20
Voatakaboka	2.04	0.00	2.04
Hazomainty	0.00	1.93	1.93
Apaliala	0.00	1.72	1.72
Tomenjy	0.00	1.63	1.63
Vavaporetaka	0.00	1.52	1.52
Anakatsimba	1.46	0.00	1.46
Vahivoraka	0.00	1.41	1.41
Famakilela	0.00	1.22	1.22
Vahiharotra	0.87	0.31	1.18
Varongy	1.00	0.00	1.00
Kalamasombarika	0.00	0.84	0.84
Kimbaletaka	0.83	0.00	0.83
Kimbatenany	0.79	0.00	0.79
Solaitra	0.00	0.75	0.75
Tavolopina	0.00	0.69	0.69
Ramiavona	0.44	0.18	0.62
Kalafambakaka	0.55	0.00	0.55
Tongobivy	0.00	0.49	0.49
Velatra	0.44	0.00	0.44
Malanimanta	0.41	0.00	0.41
Guava	0.33	0.00	0.33
Hafotra	0.00	0.32	0.32

Table 3.4 continued

Tree Species	Feeding time *E. rubriventer* (%)	Feeding time *E. rufifrons* (%)	Total (%)
Kilelakomby	0.30	0.00	0.30
Masoposaina	0.00	0.29	0.29
Vatsilana	0.00	0.25	0.25
Fahitra	0.00	0.24	0.24
Tefloya	0.24	0.00	0.24
Sandramy	0.00	0.20	0.20
Rovari	0.00	0.11	0.11
Valotra	0.00	0.10	0.10
Robrary	0.00	0.09	0.09
Vahindavenona	0.00	0.07	0.07
Anambahy	0.00	0.07	0.07
Bararata small	0.00	0.06	0.06
Sirahazo	0.00	0.06	0.06
Lalona	0.00	0.03	0.03
Dendemo	0.00	0.00	0.00
Soil	0.00	0.58	0.58
Fungi	1.60	0.00	1.60

CHAPTER 4

OCCUPANCY STRONGLY INFLUENCES FAECAL MICROBIAL COMPOSITION OF WILD LEMURS

de Winter, I.I., Umanets, A., IJdema, F., Ramiro-Garcia, J., van Hooft, W.F., Heitkönig, I.M.A., Prins, H.H.T., Smidt, H.

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ABSTRACT

The microbiota of the mammalian gut is a complex ecosystem and its composition is greatly influenced by host genetics and environmental factors. In this study, we aim to investigate the influence of occupancy (i.e., geographic location of a species), species, age, and sex on intestinal microbiota composition of the three lemur species *Eulemur fulvus*, *E. rubriventer*, and *E. rufifrons*. Faecal samples were collected from a total of 138 wild lemurs across Madagascar and microbial composition was determined using next generation sequencing of PCR-amplified 16S ribosomal RNA gene fragments. In line with studies on other primate species, the predominant phyla we detected were *Firmicutes* (43±6.4% SD) and *Bacteroidetes* (30.3±5.3%). The microbial composition was strongly associated with occupancy in the *E. fulvus* population, explaining 35.7% of the total variation in microbial composition. In turn, the difference observed in the faecal microbiota between lemur species were less pronounced, as was the impact of sex and age. Our findings show that occupancy had the strongest influence on intestinal microbiota of congeneric lemur species. This suggests the adaptation of microbiota in lemurs to differences in forest composition, climate variations, and corresponding diet in the different geographical locations of Madagascar.

INTRODUCTION

The intestinal microbiota of mammals is an integral part of an animal's body. It contributes significantly to the overall health of the host through facilitation of food digestion, modulation of its immune system, competition with pathogenic microorganisms, and the production of metabolites that are beneficial to the host (Kabat et al., 2014; Patterson et al., 2014). Expression of these beneficial properties directly correlates with microbial community diversity and composition (Clemente et al., 2012). Hence, identifying the factors and underlying processes that shape the intestinal microbiota composition is important for a better understanding of its contribution to host health. Previous studies in humans have shown that host genetics (Hansen et al., 2010), lifestyle (David et al., 2014), and food preferences (Maukonen and Saarela, 2014) contribute to shaping microbiota composition of an individual within a population. Intestinal microbiota composition can be distinguished between different mammalian species, suggesting co-evolution and adaptation of animals and their microbes (Ley et al., 2008; Moeller et al., 2016). It is not clear, however, to what extent the specific host species and environmental factors influence intestinal microbial composition under natural conditions among closely related species dwelling in different biogeographical regions.

Wildlife microbiota have received less attention in comparison to the microbiota of humans and rodent model animals. However, data collected from species in the wild can provide complementary information that contributes to our understanding of processes that shape the intestinal microbiota. For instance, studies that highlight similarities and differences in microbiota between humans and other *Homininae* species (Ellis et al., 2013; Moeller et al., 2014; Schnorr et al., 2014) provided new insights into the evolution of microbiota, suggesting adaptation of human microbes to an animal protein-based diet. Studies on microbiota composition of primates that are evolutionarily more distant from humans, such as yellow baboons (*Papio cynocephalus*) (Ren et al., 2015), black howler monkey (*Alouatta pigra*) (Amato et al., 2015), black and white colobus (*Colubus guereza*), red colobus (*Piliocolobus tephrosceles*), and red-tailed guenon (*Cercopithecus ascanius*) (Yildirim et al., 2010), revealed that microbiota composition of these primates is highly variable, also intra-individually, and mostly depends on the available diet. Correspondingly, the food availability and therefore the diet of a wild animal directly depends on geologic and climatic circumstances, as well as the flora and fauna of an area.

This also holds for wild lemurs in Madagascar, for which several studies showed variation in feeding patterns and diet when comparing areas with a different forest composition (Balko and Underwood, 2005), as well as differences in seasonality (Overdorff, 1993; Overdorff et al., 1997). One study compared the microbiota composition of sympatric wild *Lemur catta* and *Propithecus verreauxi* across dry and

wet seasons and showed that microbiota of both lemur species is variable between individuals and can be dynamic throughout the year (Fogel, 2015). Researchers observed differences in microbial composition between wild and captive *L. catta* as well as between wild populations of *L. catta* and *P. verrauxi*, only with respect to relative abundance of specific microbial groups (McKenney et al., 2015). Wild rufous mouse lemurs (*Microcebus rufus*) showed differences in gut microbial diversity with age and sites. Furthermore, microbial composition and richness were influenced by site, sex, and year, whereas temporal trends within a year were weak (Aivelo et al., 2016).

The above mentioned studies of lemur microbiota were focused on a single lemur species (Aivelo et al., 2016), two sympatric lemur species dwelling in the same area (Fogel, 2015), or captive lemurs of different species (McKenney et al., 2015). Taken together, these studies showed that lemurs harbour complex intestinal microbiota, the composition of which fluctuates over time among and within individuals, and is influenced by season, captivity, age, site of sampling, and sex. In our study, we focused on microbiota of three closely related *Eulemur* species: *Eulemur fulvus*, *E. rufifrons*, and *E. rubriventer*. These species are exposed to large variations in climate conditions and biogeography. To the best of our knowledge, this is the first comparative study of intestinal microbiota composition of multiple wild lemur species across Madagascar. In addition to an explorative assessment of the most important features of intestinal microbial composition in these species, we addressed to what extent occupancy (i.e., geographic location of a species), host species, sex, and age influence lemur intestinal microbial composition and which of these factors contribute most strongly to intestinal microbiota differentiation in wild lemurs. To this end, we hypothesised that intestinal microbial composition is similar among congeneric lemur species and that occupancy has the strongest influence on intestinal microbiota differentiation.

METHODS

STUDY DESIGN

We selected faecal samples (N = 138) from wild lemurs across Madagascar from April to July 2014 (Fig. 4.1). To investigate the effect of lemur occupancy on intestinal microbiota, we compared *E. fulvus* samples from three geographic locations and *E. rufifrons* samples from two geographic regions with each other. To assess the influence of different species, *E. rubriventer* samples collected in Ranomafana National Park (NP) were compared to *E. rufifrons* samples from the same area. The effect of age and sex was estimated based on all samples.

STUDY SITES

Madagascar experiences strong variation in climate conditions, resulting in different vegetation zones across the island (Fig. 1.1) (Irwin et al., 2010). We studied the effect of environmental factors on lemur microbiota composition at five sites across Madagascar (Fig. 4.1). Kirindy Forest (20°07'S, 44°67'E, 722 km^2) and Ankarafantsika NP (16°25'S, 46°80'E, 1350 km^2) consist of dry deciduous forest and are located on the western- and north-western side of Madagascar respectively (Goodman and Benstead, 2005). Kirindy Forest is characterised by pronounced seasonality. This forest consists mostly of deciduous trees with adaptations to water stress (Lewis and Bannar-Martin, 2012). Ankarafantsika NP is a mosaic of floristically heterogeneous dry deciduous forests dissected by small valleys with abundant Raffia palms (Ganzhorn and Schmid, 1998; García and Goodman, 2003; Rakotonirina, 1996; Sorg et al., 2003). Ranomafana NP is located in southeastern Madagascar (21°16'S, 47°20'E) and encompasses approximately 435 km^2 of montane moist forest, ranging from altitudes of 500 m up to 1500 m, and receives an average of 3000 mm rainfall per year (Wright et al., 2012). The rainfall in Ranomafana NP differs between the wet-warm season (December through March, 482 to 1170 mm per month) and dry-cold season (April through November, 55 to 513 mm per month) (Atsalis, 2000). Andasibe Mantadia NP (155 km^2) is located at the eastern side of Madagascar (18°92'S, 48°42'E) and is also characterised by a relatively wet rain forest. Nosy Tanikely (13°28'S, 48°14'E) is an island in the north-east of Madagascar and is covered with tropical vegetation. This island comprises less than 0.3 km^2 and is located between Nosy Be (8 km) and the mainland of Madagascar (13 km). Elevation ranges from 0 to 47 meters above sea level (Köhler et al., 1997). The island's vegetation consists of low forest with planted banana and mango trees, surrounded by a sandy shore with large rock formations (I. de Winter, *personal observation*).

STUDY SPECIES

This study focused on three *Eulemur* species: the red-fronted brown lemur (*E. rufifrons*), the common brown lemur (*E. fulvus*), and the red-bellied lemur (*E. rubriventer*). These species are morphologically alike and are frugivorous, although they may include other food sources, such as leaves and invertebrates, in their diet (Overdorff, 1996; Pyritz et al., 2011; Sato, 2013; Sussman, 1974). The main difference in social organisation between the *Eulemur* species is their group size. *Eulemur rufifrons* and *E. fulvus* live in multi-male, multi-female groups from four to 18 individuals (Johnson et al., 2005; Pyritz et al., 2011), whereas *E. rubriventer* lives in small monogamous groups from two up to five individuals (Tecot 2008, 2010; Tecot et al., 2016).

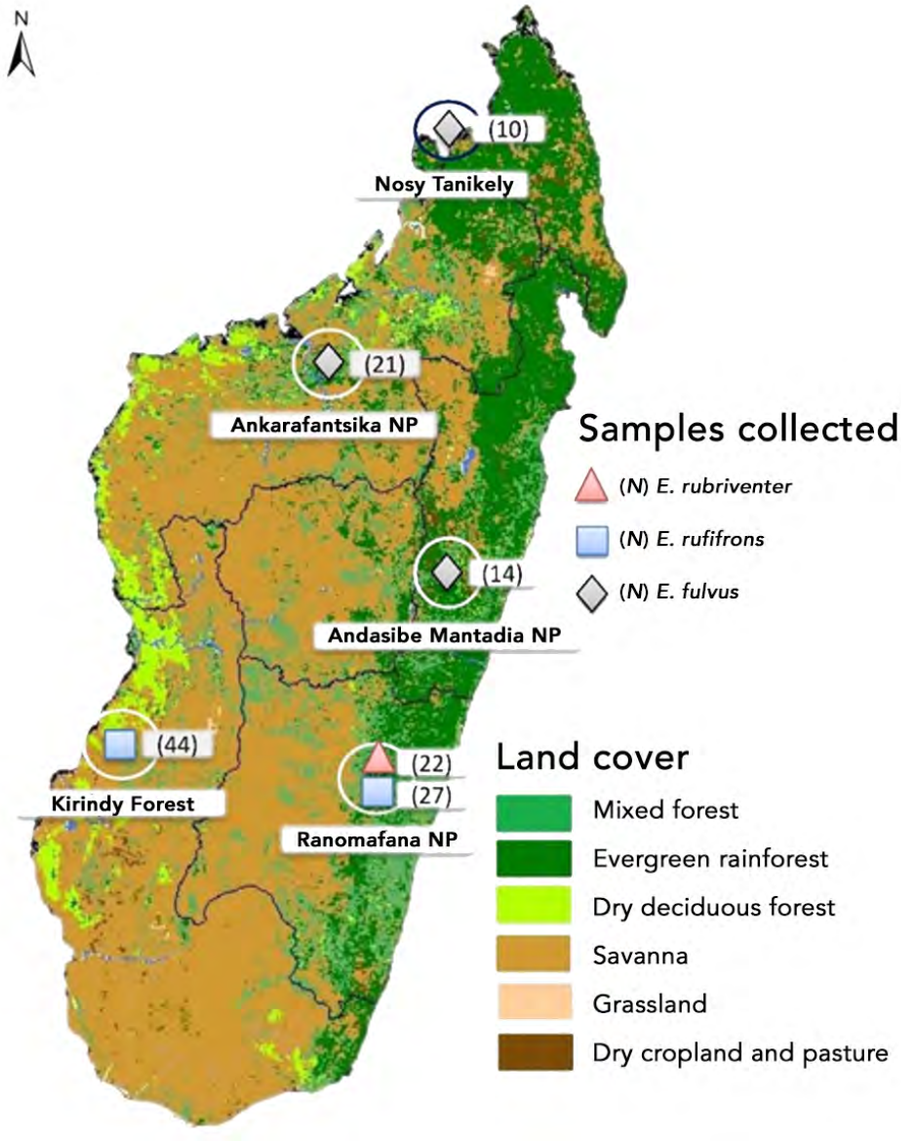

Figure 4.1: Lemur faecal sample collection areas across Madagascar. The map shows the main types of land cover and vegetation, was adapted from www.wildmadagascar.org, and was produced with data taken from the FAO Country Profiles and Mapping Information System (The United Nations Food and Agricultural Organization; FAO 2004). Faecal samples were collected at five geographical locations across the island from *E. rufifrons* (two sites), *E. fulvus* (three sites), and *E. rubriventer* (one site). (*N*) = number of samples.

SAMPLING AND DATA COLLECTION

Immediately after defecation, we non-invasively collected fresh faecal samples (3 to 4 g) from individual lemurs. We recorded lemur species, age, and sex. Within 12 h after collection, the samples were stored at ambient temperature in sterile plastic tubes that were prefilled with 5 ml of 70% ethanol until further analyses at the Laboratory of Microbiology, Wageningen University, The Netherlands. All samples included in this chapter were taken in compliance with the laws of the Government of Madagascar

and no animal experiments were involved. Sample collection and export was approved by the trilateral commission (CAFF/CORE) in Madagascar (permits 297/13 and 143/14/MEF/SG/DGF/DCB.SAP/SCBSE).

DNA EXTRACTION

Samples collected in Ranomafana NP were processed using a modified protocol (Yu and Morrison, 2004), with specific, previously described modifications (Salonen et al., 2010). Faecal material was air-dried for 15 to 20 min in a fume hood to remove ethanol from the samples. Subsequently, 0.1 to 0.17 g of dried samples was added into double autoclaved screw-cap tubes containing 0.3 g of 0.1 mm zirconia beads, three pieces of 2.5 mm glass beads, and 700 μl of lysis buffer (500 nM NaCl, 50 mM Tris-HCl (pH 8), each containing 50 mM EDTA 4% SDS). Samples were treated for 3x1 min at $5.5x10^3$ movements per minute in a Precellys 24 beadbeater (Bertin technologies, France). After homogenisation, samples were incubated at 95°C for 15 min in a shaking heating block (Vartemp 56, Labnet International, Edison, NJ, USA) at 100 rpm and then centrifuged at 4°C for 5 min at 13,000 rpm. Clean supernatants were transferred into 2 ml tubes. Next, we added 300 μl of fresh lysis buffer in the same tubes to the pellets, bead beating and incubation steps were repeated, and the newly collected supernatant was pooled with the previously collected supernatant. Subsequent steps were performed according to the original protocol (Yu and Morrison, 2004).

Samples collected in Andasibe NP, Kirindy Forest, Ankarafantsika NP, and Nosy Tanikely were extracted using an automatic system, the Maxwell® 16 Research Instrument (Promega, Madison, USA), and the corresponding RNA extraction kit according to manufacturer's instructions. To improve DNA yield, samples preserved in 70% ethanol were rehydrated through a series of ethanol solutions with decreasing proportions of ethanol in steps of 10%. For rehydration, 1.5 ml of 70% ethanol with faecal particles was transferred into a fresh 2 ml tube and centrifuged at 13,000 rpm for 5 min to separate solid fractions from the liquid. After centrifugation, part of the supernatant was replaced with the same amount of distilled water to decrease ethanol concentration by 10 percentage points, vortexed, and incubated for 10 min at room temperature. These steps were repeated until the ethanol was replaced by distilled water. Cell disruption and lysis was performed as described above, but we used S.T.A.R. buffer instead of lysis buffer (Roche Molecular Systems, USA).

We determined DNA quality and concentration spectrophotometrically (Nanodrop Technologies, Wilmington, USA). Comparison of the two DNA extraction methods mentioned above, using human faecal samples, indicated that both methods delivered DNA of essentially equal quality, resulting in comparable results with respect to microbial composition based on analyses with the Human Intestinal Tract Chip (HITChip), a DNA oligonucleotide microarray targeting human intestinal

microbiota (Heikamp-de Jong and Hartman, *personal communication*).

16S RRNA AMPLIFICATION AND LIBRARY PREPARATION

After DNA extraction, regions V1-V2 of the 16S rRNA genes were amplified using an in-house two-step PCR protocol. In the first step, regions of interest were amplified using the following primers: 27F–DegS: GTTYGATYMTGGCTCAG (van den Bogert et al., 2011) and an equimolar mix of 338R–I: GCWGCCTCCCGTAGGAGT (Daims et al., 1999) and 338R–II: GCWGCCACCCGTAGGTGT (van den Bogert et al., 2013), with attached UniTag I (forward) and II (reverse) linkers (I – GAGCCGTAGCCAGTCTGC; II - GCCGTGACCGTGACATCG) (Tian et al., 2016). The PCR mix for one reaction at step one contained 10 μl of 5x HF buffer, 1μl dNTPs (10 μM), 1U of Phusion Hot start II DNA polymerase (2U/μl), 31.5 μl of nuclease free water, 2.5 μl of forward (10 μM) and 2.5 μl of reverse primers (10 μM), and 40 ng of DNA template. Amplification was performed in a LabCycler Gradient (SensoQuest, Germany) programmed for initial denaturation at 98°C for 30 sec and 25 cycles of denaturation at 98°C for 10 sec, annealing at 56°C for 20 sec and extension at 72°C for 20 sec, followed by final extension at 72°C for 10 min. After amplification, the success of the PCR reaction was checked visually by agarose gel electrophoresis, considering the amount and size of the amplicon as quality parameters.

Amplicons were subsequently used as template for a second PCR for the introduction of sample-specific barcodes, using individual barcode primers for each sample. In total, we used 48 pairs of forward and reverse barcode primers that target UniTag1 and UniTag2 sequences introduced during the first PCR, respectively, and that were appended with sample-specific barcodes. Composition of PCR reagents and cycling conditions were as described for the first PCR, with 10 μl of PCR products from the first step as template. Reactions were performed in a final volume of 100 μl. PCR products were purified and concentrated using magnetic beads (MagBio, Switzerland) according to the HighPrep protocol with adaptation for 2 ml tubes. Purified products were quantified using the Qubit dsDNA BR Assay Kit (Life Technologies, USA) following the manufacturer's protocol. PCR products were pooled in equimolar amounts into libraries of 48 samples each and sequenced on an Illumina MiSeq platform in 300 bp paired end mode at GATC Biotech (Constance, Germany).

DATA PROCESSING AND STATISTICAL ANALYSIS

Initial analysis of raw 16S rRNA gene sequencing data was performed using the NG-Tax pipeline (Ramiro-Garcia et al., 2016). Sequences were separated into sample-specific bins based on the barcodes, after initial filtering of paired-end libraries to contain only read pairs with perfectly matching barcodes. Operational Taxonomic Units (OTUs) were defined using an open reference approach and taxonomy was assigned using a SILVA

16S rRNA gene reference database (Quast et al., 2013). Microbial composition plots were generated using a workflow based on Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 (Caporaso et al., 2010).

Reads assigned to OTUs of plant origin, such as chloroplast and plant mitochondrial DNA, were removed from the dataset used for downstream analyses. OTU counts were normalised using cumulative sum scaling (CSS) (Paulson et al., 2013). To get an overview of species composition, we visualised a normalised OTU matrix and calculated the relative contribution based on normalised OTU numbers per taxa. Median values of taxa relative abundance in a group of samples were used to compare groups. The OTU matrix was filtered to exclude OTUs that were only present in a small number of samples. More specifically, for each dataset, OTUs were removed that were present in less than five samples (50% of the smallest group size).

Measures of alpha and beta diversity and initial multivariate analysis using principal coordinate analysis (PCoA) were performed on the rarefied matrix (depth -1650 observations), using weighted and unweighted UniFrac as distance measures as implemented in QIIME. We used Kruskal-Wallis tests when comparing more than two groups to explore differences in relative abundances of OTUs between individual samples. We used nonparametric *t*-tests with 500 Monte Carlo permutations in case of comparisons of two groups, using normalised, summarised, and filtered OTU tables. False discovery rate (FDR) correction of *P*-values was used to reduce the chance of type I statistical errors, when multiple statistical hypotheses were tested. To identify strength and statistical significance of sample groupings with weighted and unweighted UniFrac as distance measures, we applied the Adonis-test as implemented in the R package 'vegan'. Canoco 5.0 was used for multivariate statistical analysis and visualisation of correlations between microbial composition of samples and explanatory factors. Redundancy analysis (RDA) was performed as described previously (Šmilauer and Lepš, 2014). As input dataset for RDA we used the taxonomy summary table at genus level after removal of taxa that were present in less than eight samples (applied for each dataset individually). The significance of observed community variations was evaluated using a Monte Carlo permutation test.

To identify how microbial species were correlated with investigated factors, such as area or host species, we used the LefSe (Linear discriminant analysis of Effect Size) algorithm for biomarker identification (Segata et al., 2011). We processed the data using tools developed by the Huttenhower laboratory, implemented in the Galaxy environment. Preparation of input data and analysis were performed according to the standard workflow, using default settings (0.05 - alpha value for the factorial Kruskal-Wallis test among classes, the threshold on logarithmic LDA score for discriminative features was 2.0, and the strategy for multi-class analysis was 'all-against-all').

After raw data processing and initial analysis, samples were organised into five

sets (samples are used in several sets), allowing us to perform separate analyses and statistics while focusing on a particular research question: (1) an initial set of all 138 samples; (2) samples obtained from *E. fulvus* (*N* = 45) from three different areas (Andasibe NP (*N* = 14); Ankarafantsika NP (*N* = 21); Nosy Tanikely (*N* = 10)); (3) a set of samples from *E. rufifrons* collected in Kirindy Forest (*N* = 44); (4) samples from *E. rufifrons* (*N* = 27) and *E. rubriventer* (*N* = 22) collected in Ranomafana NP (total *N* = 49); (5) samples from *E. rufifrons* collected in two different areas (Ranomafana NP (*N* = 27) and Kirindy Forest (*N* = 44)). To determine to what extent occupancy (i.e., area of habitation) can explain the observed variation in faecal microbiota, we analysed three datasets: with all samples, with samples from *E. fulvus,* and with samples from *E. rufifrons*. The dataset containing all samples allowed us to identify the influence of occupancy among all other variables such as host species, sex, and age. The lemur species *E. fulvus* was sampled at three different locations and *E. rufifrons* in two, allowing us to more specifically address variation in faecal microbiota composition found within one species exposed to different environmental conditions. Datasets generated in this study are available in the public read archive EBI, study name 'Area of habitation strongly influences faecal microbial composition of wild lemurs', with accession number PRJEB20007.

RESULTS

In this study, we analysed the faecal microbiota of 138 individuals belonging to three different *Eulemur* species, using Illumina MiSeq sequencing of PCR-amplified 16S ribosomal RNA gene fragments covering the V1-V2 variable region. In total, we obtained 6,220,515 reads, ranging from 1652 to 178,522 reads per sample (r/s) with a median of 36,092 r/s. Obtained reads were assigned to 1053 OTUs using NG-Tax, an in-house developed pipeline (Ramiro-Garcia et al., 2016). Across all samples, OTUs belonged to twelve bacterial phyla, i.e., *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Candidatus Saccharibacteria* (TM7), *Cyanobacteria*, *Firmicutes*, *Lentisphaerae*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, *Tenericutes*, and *Verrucomicrobia*. The fraction OTUs that were not assigned to any taxonomic level varied from 2.6 to 16.7% with an average of 9.3±2.5% SD in all analysed samples. Predominant phyla, regardless of lemur species and sampling location, were *Firmicutes* 43.3±6.4%, *Bacteroidetes* 30.3±5.3%, *Cyanobacteria* 5.2±3.3%, and *Proteobacteria* 7.4±3.1%. At the genus level, a total of 59 taxa were identified, fifteen of which had an average relative abundance across all samples of more than 1% and comprised more than 80% of all sequences, and only three out of fifteen were assigned to known genus. Overall, 34% of all OTUs could be assigned at genus level, and 63% at family level. Phylogenetic clustering based on relative abundance at the genus level showed that the most abundant genera could be clustered into two groups: one group consisting of the two most abundant genera (unidentified genus (UG) 1 (*Clostridiales*) 24.9%±5.4%; UG_2 (*Bacteroidales*) 14.9±3.6%); and another group consisting of another five genera (UG_3 (*Prevotellaceae*) 7.7±2.3%; UG_4 (C*yanobacteria*) 5.2±3.3%; UG_5 (*Bacteroidaceae*) 4.6%±2.4%; UG_6 (*Lachnospiraceae*) 4.9%±2.8%; UG_7 (*Ruminococcaceae*) 4.2%±2%).

SPECIES EFFECT

In order to address the influence of lemur species on observed variation in microbial composition, two different datasets were analysed: the entire dataset of 138 samples, and the dataset with the samples collected in Ranomafana NP. The latter allowed us to minimise the influence of explanatory variables other than host species. We found that samples from *E. fulvus* (N = 45) showed a significantly lower alpha diversity (P = 0.003) in comparison with samples taken from *E. rufifrons* (N = 22). The relative abundance of *Proteobacteria, Lentisphaerae, Synergistetes, Bacteroidetes, Actinobacteria, Firmicutes, Tenericutes*, and *Candidatus Saccaribacteria* (TM7) differed among studied lemur species (FDR-corrected $P < 0.012$, Fig. 4.2A). At genus level, 26 taxa differed in relative abundance (corrected $P < 0.036$), with some being only present within one lemur species. The genera *Anaeroplasma* and UG_8 (*Desulfovibrionaceae*) were only found in samples belonging to *E. rufifrons*, albeit with

relative abundances below 1%. Eight genera were identified by LefSe analysis as potential biomarkers for the different lemur species: *Bacteroides* and *Phascolarctobacterium* were identified as microbial biomarkers for *E. rufifrons*; UG_6 (*Lachnospiraceae*), *Campylobacter*, UG_9 (*Synergistales*), UG_10 (*Clostridiales*) and UG_11 (*Xanthomonadales*) were biomarkers for *E. rubriventer*; and UG_12 (*Anaeroplasmatales*) was associated with *E. fulvus* (Fig. 4.2B). No clear grouping of samples by lemur species was observed based on either weighted or unweighted UniFrac distances. This was confirmed by the Adonis-test that revealed only a weak linear correlation between samples, with an R^2 of 0.11 and 0.13 for weighted and unweighted distances, respectively. RDA with lemur species as the only explanatory variable showed that this variable significantly ($P = 0.008$) contributed to the observed variation in faecal microbiota composition (Fig. 4.3A). Furthermore, when comparing the faecal microbiota of *E. rufifrons* and *E. rubriventer* in Ranomafana NP, RDA analysis showed that, although 'lemur species' was a significant explanatory variable ($P = 0.024$), it only explained 6.8% of the observed variation in microbial community composition (Fig. 4.3B). All phyla observed in the full dataset were also present in Ranomafana NP, and only the phylum *Firmicutes* showed near significant differences in relative abundance between *E. rufifrons* (44.3±7.1%) and *E. rubriventer* (39.7±4.7%, $P = 0.008$; corrected $P = 0.1$). Based on uncorrected *P*-values, the observed difference in relative abundance of *Firmicutes* was significant. When we corrected for false discovery, this was no longer significant, and thus, the observed difference would have to be confirmed in larger studies. At genus level UG_13 (*Porphyromonadaceae*), UG_5 (*Bacteroidaceae*), and UG_19 (*Bacillales*) differed in relative abundance when comparing microbial composition in both lemur species (corrected $P < 0.04$). Similar to the dataset including all samples, no separation or grouping was observed among samples from Ranomafana NP in weighted or unweighted UniFrac matrix-based PCoA plots ($R^2 = 0.06$ and $R^2 = 0.05$ for unweighted and weighted distance matrices, respectively; data not shown).

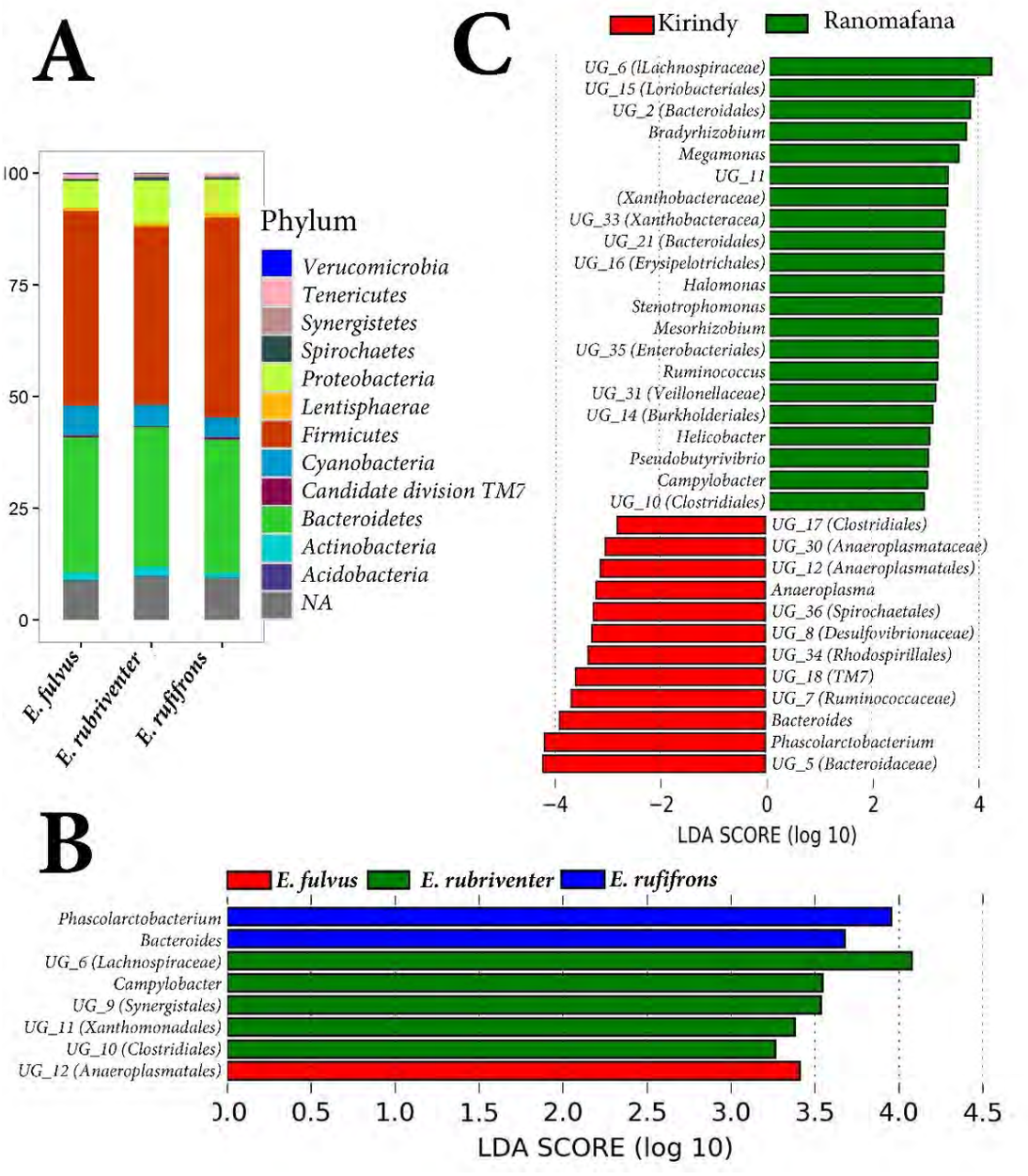

Figure 4.2: Differences in bacterial composition between lemur species (*E. fulvus*, *E. rubriventer*, and *E. rufifrons*). A) relative abundance at the phylum level of faecal microbiota of the different lemur species using the complete dataset ($P = 0.008$); B) taxa identified by LefSe as potential biomarkers for the discrimination of studied *Eulemur* species. LDA - linear discriminant analysis; C) taxa identified by LefSe as potential biomarkers for the discrimination of faecal samples taken in Ranomafana NP and Kirindy Forest.

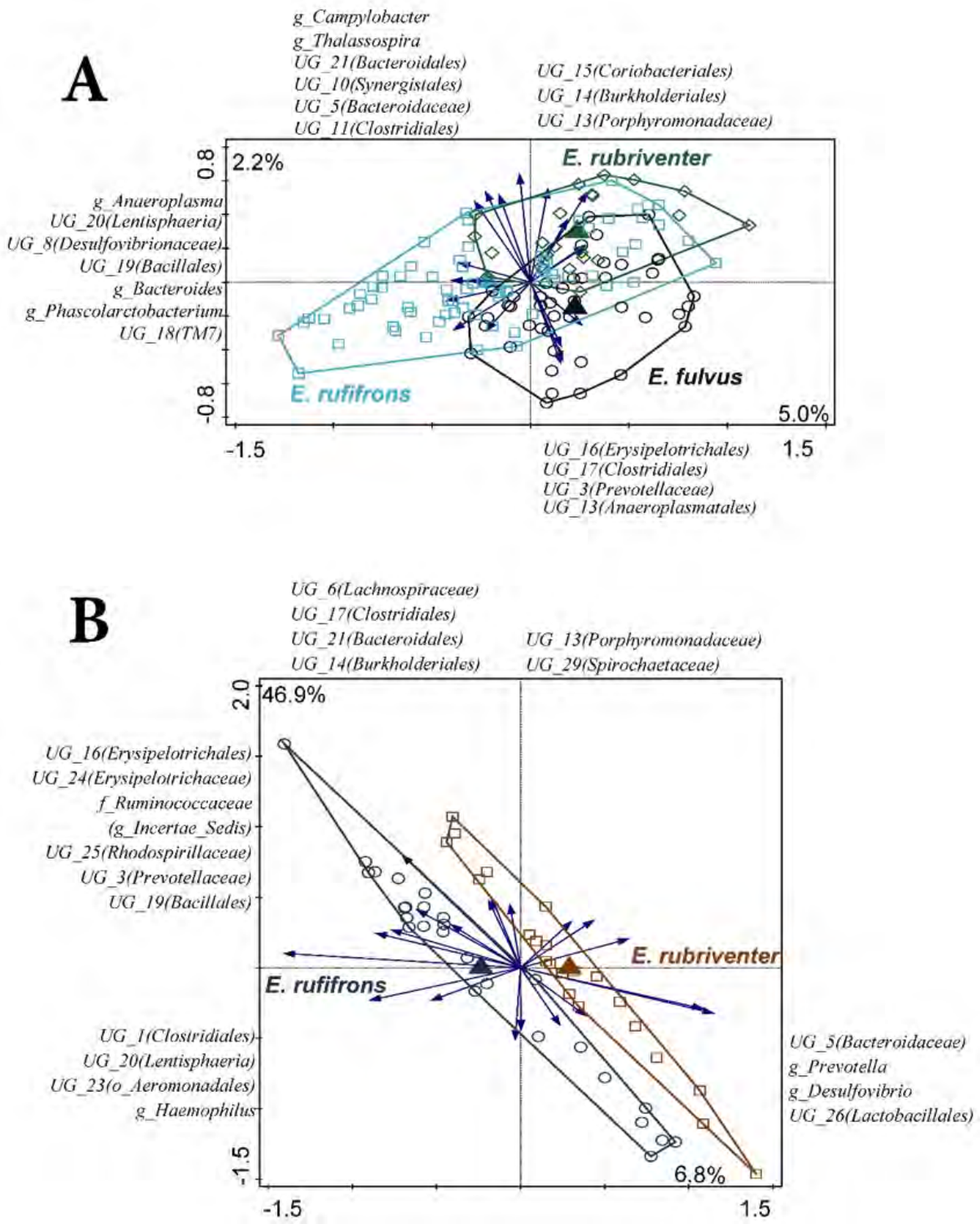

Figure 4.3: Ordination triplots based on RDA with lemur species as explanatory variables. A) For all lemur species, 7.2% of the variation is captured by the first two canonical axes; B) for lemur species in Ranomafana NP, 6.8% of variation is captured by the canonical axis, and both host species significantly (P = 0.002) contributed to explaining the observed variation in microbiota composition.

LEMUR OCCUPANCY

Samples from Nosy Tanikely (*N* = 10) had the lowest alpha diversity when compared to all other areas (*P* = 0.01). The relative abundances of 10 out of 12 phyla, except for *Bacteroidetes* and *Acidobacteria*, were different (corrected *P* < 0.026) between sampling sites. It should be noted that members of the phylum *Acidobacteria* were found in only a few samples (10 out of 138). Furthermore, at genus level, 54 out of 59 taxa that were observed in more than five samples, showed significant differences in relative abundance between areas (corrected *P* < 0.05). Among these genera, some were only found within Kirindy Forest, namely *Anaeroplasma, Rhizobium*, and UG_8 (*Desulfovibrionaceae*). Members of the genus *Bacteroides*, UG_13 (*Anaeroplasmatales*), UG_17 (*Clostridiales*), and UG_30 (*Anaeroplasmataceae)* were found exclusively in samples from the relatively dry areas Kirindy Forest and Ankarafantsika NP. The most abundant genus (UG_1 (*Clostridiales*)) did not vary significant between areas. Samples showed a slight visual grouping according to area in PCoA plots based on weighted UniFrac distances (R^2 = 0.29), with a higher group separation being observed in the case of unweighted UniFrac (R^2 = 0.34, Fig. 4.4A, B). Furthermore, constrained analysis (RDA) showed that all areas included as explanatory variable significantly (*P* < 0.05) contributed to the observed variation in faecal microbiota composition (Fig. 4.5A).

For *E. fulvus*, eight out of twelve phyla (*Tenericutes, Cyanobacteria, Spirochaetes, Lentisphaerae, Firmicutes, Candidatus Saccaribacteria, Proteobacteria*, and *Actinobacteria*) differed in relative abundance between areas (corrected *P* < 0.01). In line with the extensive differences observed at the phylum level, 34 out of 55 detected genera showed significant differences in relative abundance between areas. UG_17 (*Clostridiales*), UG_35 (*Enterobacteriales*), UG_29 (*Spirochaetaceae*), UG_12 (*Anaeroplasmatales*), and the genus *Bacteroides* were found only in the samples from Ankarafantsika NP. UG_31 (*Veillonellaceae*) was found exclusively in the samples from Andasibe NP, and *Mesorhizobium* only in Nosy Tanikely. Several genera were absent in one of three areas: UG_19 (*Bacillales*), *Helicobacter*, and *Thalassospira* were not detected in samples taken in Nosy Tanikely, whereas *Pseudobutyrivibrio* was not found in Andasibe. Samples from *E. fulvus* clustered into three groups, correlating with the three different sampling sites based on the relative abundance of bacterial genera. Furthermore, samples formed separated groups in PCoA plots based on weighted and unweighted UniFrac distances (R^2 = 0.35 and R^2 = 0.41, respectively; Fig. 4.4C, D). RDA showed that among all factors only occupancy significantly (*P* = 0.002) contributed to explaining observed differences in faecal microbial composition of *E. fulvus*, with Ankarafantsika NP having the highest explanatory value (19.9%) (Fig. 4.5B).

For *E. rufifrons*, the relative abundance of the phyla *Actinobacteria, Candidatus*

Saccaribacteria, and *Proteobacteria* was different between the two locations where this lemur species was found, i.e., Kirindy Forest and Ranomafana NP (corrected $P < 0.009$). In total, 26 genera were different in relative abundance when comparing both locations (corrected $P < 0.047$). Members of the genus *Bacteroides* were completely absent from the samples collected from Ranomafana NP, while their mean relative abundance was 1.4±1% among samples collected from Kirindy Forest. All remaining genera that were found exclusively in samples collected in Kirindy Forest (UG_8 (*Desulfovibrionaceae*), UG_12 (*Anaeroplasmatales*), and *Anaeroplasma*) had relative abundances below 0.3% . The genus *Phascolarctobacterium* was found in all samples from Kirindy Forest (mean abundance 3±1.3%), but only in 12 out of 27 samples (average abundance 0.2±0.2%) from Ranomafana NP. UG_21 (*Bacteroidales*, 0.2±0.4%), *Mesorhizobium* (0.1±0.2%), UG_33 (*Xanthobacteraceae*, 0.4±0.2%), *Stenotrophomonas* (0.1±0.2%), UG_32 (*Rhizobiales*, 0.3±0.9%), and UG_10 (*Clostridiales*, 0.1±0.1%) were found exclusively in Ranomafana NP, albeit not in all samples collected in that area and at low relative abundances. Additional differences included UG_5 (*Bacteroidaceae*) (6.2±2.2% in Kirindy Forest vs 3.3±2% in Ranomafana NP) and UG_6 (*Lachnospiraceae*) (6.2±2.3% in Ranomafana NP vs 2.9±1.9% in Kirindy Forest). Twenty-one genera for Ranomafana NP and twelve for Kirindy Forest were identified by LefSe as microbial biomarkers (Fig. 4.2C). Multivariate analyses supported the separation of samples according to sampling location, with a clear grouping being observed in PCoA plots based on both weighted ($R^2 = 0.19$) and unweighted ($R^2 = 0.23$) UniFrac distance matrices (Fig. 4.4E, F). Furthermore, RDA showed that from all explanatory variables (area, age, and sex) only occupancy significantly contributed to explaining the observed variation in microbial community composition ($P < 0.004$), with both areas (Kirindy Forest and Ranomafana NP) explaining 8.2% of variation (Fig. 4.5C).

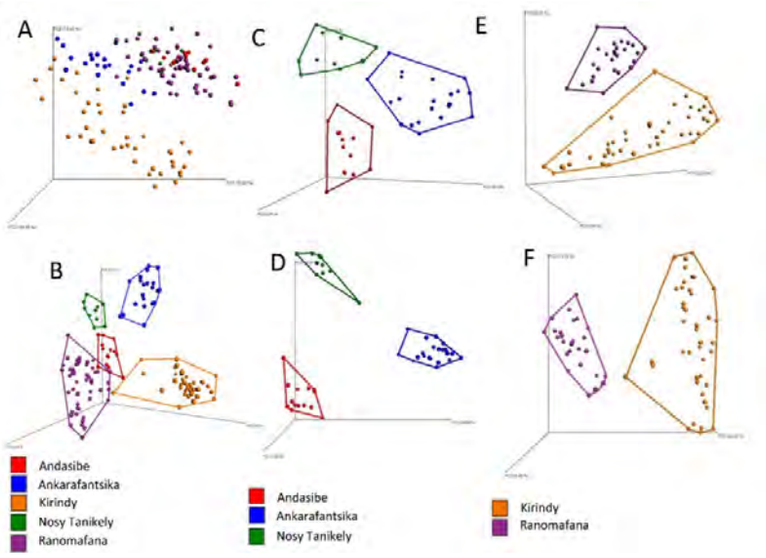

Figure 4.4: Principal coordinate analysis (PCoA) on lemur occupancy. Three dimensional (first tree PCoA axes) plots based on weighted (A, C, E) and unweighted (B, D, F) UniFrac distance matrices. Samples are represented by dots, colour-coded by sampling location. Plots contain all samples (A, B), or are species specific (*E. fulvus* C, D; *E. rufifrons* E, F).

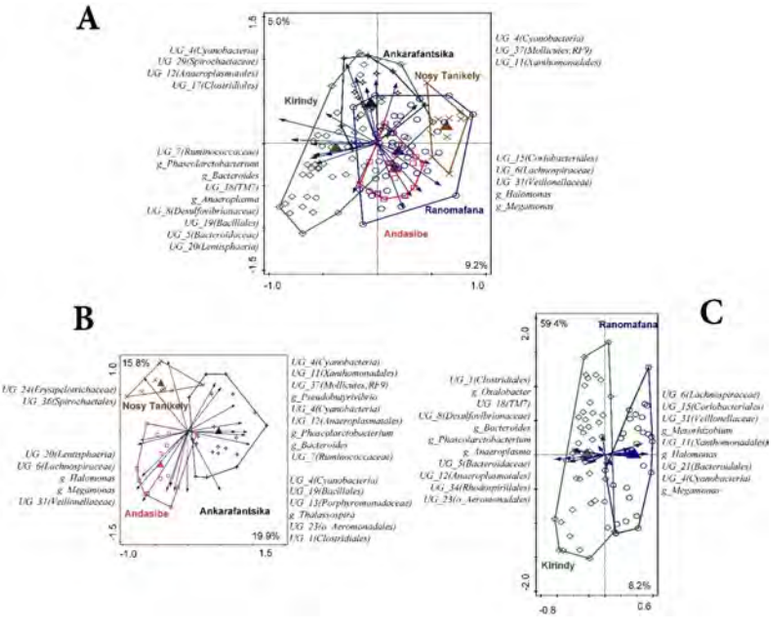

Figure 4.5: Ordination triplot based on RDA with areas of sampling as explanatory variables. A) considering all lemur species, the variation was captured by the first two canonical axes, and a statistically significant effect of areas as explanatory factors was observed; B) for *E. fulvus*, 35.7% of variation was captured by the first two canonical axes, and all three areas significantly contributed to explaining the observed variation in microbiota composition ($P = 0.002$); C) For *E. rufifrons*, 8.2% of variation was captured by the canonical axis. The variable 'occupancy' was statistically significant ($P = 0.004$) as a conditional effect.

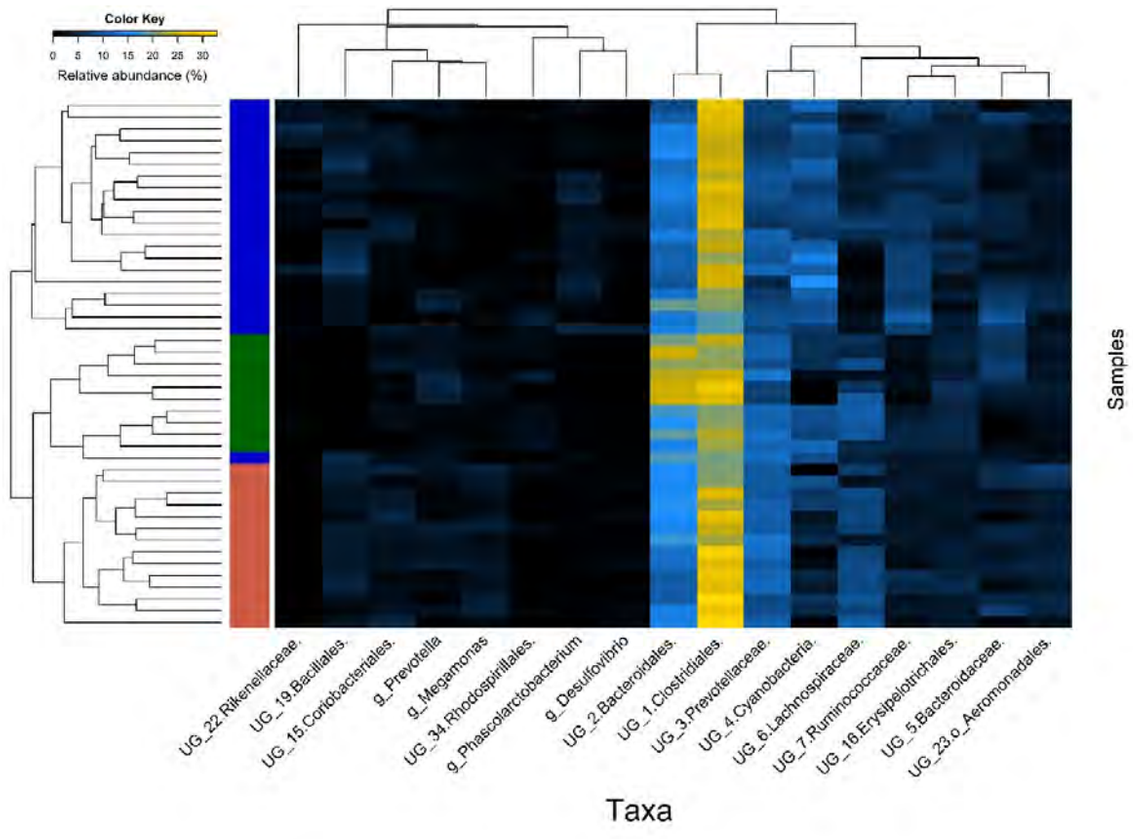

Figure 4.6: Heatmap of relative microbial abundance at genus level for *E. fulvus*. Samples placed on the y-axis and genera with relative abundance more than 2.5% across the dataset on the x-axis. The yellow colour indicates high relative abundance values, while dark blue indicates low relative abundance values. Sample clustering and the dendrogram were produced using the Bray-method as implemented in the 'vegan' R package. The side bar (left) indicates the sample collection: blue (above) – Ankarafantsika NP; green (mid) – Nosy Tanikely; red (below) – Andasibe NP.

SEX AND AGE

Influence of sex and age on the faecal microbiota composition was investigated using the complete dataset. No significant differences were observed in microbiota between samples collected from males and females in any of the datasets at phylum or genus level, and no grouping was observed with multivariate analysis using PCoA and RDA (data not shown). Similarly, no significant variation in relative abundance of detected phyla was observed between age groups, and only the relative abundance of the genus *Phascolarctobacterium* (corrected $P = 0.01$) differed among age groups when considering all samples. Multivariate analysis (RDA and PCoA) confirmed that age did not significantly contribute to explaining the variation in faecal microbiota composition.

DISCUSSION

We characterised the faecal microbiota of three frugivorous *Eulemur* species and assessed to what extent the naturally occurring variation in intestinal microbiota composition is associated with occupancy, species, age, and sex of individuals. Findings presented here showed that the gut microbial community of these animals is dominated by members of the phylum *Firmicutes* and to a lesser extent *Bacteroidetes*. It has previously been reported that predominance of *Firmicutes* or *Bacteroidetes* is different among animal species and mostly correlated with dietary composition and taxonomic lineage of a given species (Ley et al., 2008). Our results confirmed the high proportion of *Firmicutes* that was previously observed in other species of frugivorous and omnivorous primates (Gomez et al., 2015; Ochman et al., 2010), including humans, despite the fact that phylogenetically, lemurs are one of the most distinct and ancient groups within the order Primates (Ni et al., 2013). In particular, human studies showed that the *Firmicutes* to *Bacteroidetes* ratio is not static and can be largely influenced by the presence of carbohydrates in the diet, although it is not clear which of the two phyla has a leading role as key degrader of complex carbohydrates in the human intestine. For example, on the one hand, an increase in relative abundance of *Firmicutes* was correlated with the consumption of whole grains and total carbohydrate intake (Martínez et al., 2013) and several species belonging to this phylum are viewed as key degraders of resistant starch (Ze et al., 2012). On the other hand, it has been shown that the depletion of *Firmicutes* and increase in *Bacteroidetes* in African children from a rural area in comparison with European children was related to the consumption of a traditional African diet rich in fibres and polysaccharides (De Filippo et al., 2010). Such seemingly conflicting evidence might be related to the high phylogenetic and functional diversity within both phyla, including a large number of fibre- and carbohydrate-degrading species. Consequently, we speculate that specific aspects of the diet of lemurs will result in a shift of the *Firmicutes* to *Bacteroidetes* ratio, the direction of which might not be predictable based on general characteristics of the diet. Our study showed that a large fraction of *Firmicutes* associated sequences was assigned to a single genus-level taxon, UG_1 (*Clostridiales*), accounting for 24.9±5.7% of the total bacterial community (Fig 4.6). We suggest that members of this genus have an important role in intestinal function of the three *Eulemur* studied. However, due to lack of physiological and ecological data, conclusions regarding their function and role in intestinal ecology remain unknown, awaiting isolation and/or (meta)omics analyses (Gutleben et al., 2017).

SPECIES EFFECT

Notably, members of the *Proteobacteria* showed relatively high abundance (7.4±3.1%) in all three studied *Eulemur* species. In humans, high relative abundance of this phylum (9.7% to 14.9%) has been associated with gastric bypasses, metabolic disorders, inflammation, and cancer, whereas its relative abundance in healthy individuals amounts to only about 4.5% (Shin et al., 2015). However, previous research showed host species related differences in abundance of *Proteobacteria* among primates. For instance, it was observed that faecal samples of humans and chimpanzees had similar relative abundances of *Proteobacteria* (1% and 1.2% , respectively), whereas in *Gorilla gorilla* samples, this phylum reached a relative abundance of 7% (Bello González et al., 2015). Furthermore, a similar relative abundance of *Proteobacteria* (9.1%) was reported in the faecal microbiota of *Lemur catta* (McKenney et al., 2015). Hence, we suggest that the high relative abundance observed in this study is not necessarily a sign of a health problem of the investigated population of lemurs, but rather a feature of the normal microbial composition of frugivorous lemurs.

We found that on average 5.2±3.3% of all reads were assigned to OTUs belonging to the *Cyanobacteria* phylum. Latest research shows that members of this phylum are indeed a genuine part of the human intestinal microbiota (Di Rienzi et al., 2013). Furthermore, the presence of this phylum was observed in previous studies that characterised the intestinal microbial composition of other primates, including *Lemur catta* (McKenney et al., 2015). In addition, we found that 66% of genus-level taxa could not be confidently classified to a particular genus in the Silva v111 database, including several of the most predominant taxa. This is in line with the limited attention that the intestinal microbiota of lemurs has received to date, and hence there is a lack of knowledge regarding specific taxa present in the intestine of these animals. Research on intestinal microbiota of other poorly studied animals showed similar findings. For example, a study found that only 28% of the observed genera in the giraffe rumen could be assigned to known taxa (Roggenbuck et al., 2014). Similar observations have even been made for less well characterised human populations. A recent study found that 22% of the total microbial community of central Tanzanian Hadza individuals could not be assigned at family and genus level, whereas this was not the case for the Italian control population (Schnorr et al., 2014).

LEMUR OCCUPANCY

To assess the role of different natural environmental factors in shaping the intestinal microbiota and how these factors relate to the influence of the different host species, we divided samples into subsets. This approach allowed us to gain a better insight into the effect of specific factors on microbiota composition, in addition to a more

generic analysis of all factors at the same time in a relatively heterogeneous dataset. The value of this approach was confirmed by the fact that for all factors of interest, a first insight into potential effects that could be obtained with the whole dataset, received additional, more robust support from the analysis of specific subsets of samples. It should also be noted that, due to the nature of wildlife sampling under natural conditions, it remains challenging to obtain balanced sample sets with equal numbers of samples in each group.

We discovered that in samples analysed in our study, the most influential factor contributing to shaping microbiota composition was the area of sampling, i.e., lemur occupancy. When we applied PCoA based on either weighted or unweighted UniFrac distances, separation into areas was obvious in all datasets. Remarkably, clustering of samples was tighter with improved separation of samples when we used unweighted UniFrac as a distance measure. This observation suggests, taking into account the nature of the UniFrac distance calculation, that the faecal microbiota of lemurs from different areas is more distinct with respect to microbial species composition than in relative abundance of prevalent taxa. Constrained multivariate analysis (RDA) confirmed that occupancy is the most influential explanatory variable with respect to the observed variation in lemur intestinal microbiota composition.

Madagascar is known to have different environmental conditions and biodiversity within relatively small areas (Wilme et al., 2006). Furthermore, sampling locations were positioned at considerable distance from each other, and were characterised by major climatic differences such as amount of precipitation and forest composition. Hence, the availability of food resources during the year, in particular fruits and flowers, is considered as the driving force that leads to differences in the intestinal microbiota. Regardless of the area of occupancy, all true lemur species are predominantly frugivores, with fruits constituting between 66 to 95 percent of their diet. Most true lemurs also supplement their diets with leaves (< 26 percent), flowers, and nectar (< 20 percent), and other less common items, like fungi, bark, and soil (Johnson, 2006). Behavioural observations and faecal analyses on several true lemur species have shown that the lemurs in the western forests, such as Kirindy Forest and Ankarafantsika NP, have a lower dietary diversity during the dry season when compared to populations living in eastern rainforests. These studies also revealed that lemurs become largely folivorous and increase the proportion mature leaves, unripe fruits, and flowers in their diet, while decreasing their feeding time on fruits during the late dry season when ripe fruits are scarce (de Winter et al., 2013; Ganzhorn, 2002; Ossi and Kamilar, 2006; Sato, 2013; Sato et al., 2014; Sorg and Rohner, 1996). In contrast, in eastern rainforests, including Ranomafana NP and Andasibe NP, fruit is a major component of the lemurs' year-round diet (Overdorff, 1993, 1996). Also in the dry season with a relatively low fruit availability, the dietary

composition of *Eulemurs* was predominantly composed of fruits (Overdorff, 1993). Surprisingly, in the tropical rainforest on the island Nosy Tanikely, relatively low microbial alpha diversity was observed in *E. fulvus*. On this island, *E. fulvus* devotes nearly all its feeding time to the abundant mango fruits and additionally some bananas that tourists feed them (I. de Winter, *personal observation*). It is therefore tempting to speculate that the lower alpha diversity in *E. fulvus* can be explained by adaptation of the microbiota to a non-diversified and sugar-rich diet. Although true lemurs consume fruit continuously throughout the year across Madagascar, the dry season in western Madagascar is associated with a large dietary change, where leaves instead of fruits become the main energy source as a reaction to the low fruit availability (Curtis, 2004; Ossi and Kamilar, 2006). As our samples were collected in the dry season, this can explain our observation that lemur occupancy is the most influential factor contributing to shaping the microbiota composition.

We also observed differences in microbiota composition related to the specific lemur species, however, these differences were of lower importance than those observed between different areas of habitation. Host genetic differences is considered a major driving force shaping the intestinal microbiota composition (Khachatryan et al., 2008) in different animal species with similar dietary habits (Moeller et al., 2014). Our study was conducted on congeneric species that by definition are genetically close (Markolf and Kappeler, 2013) and have almost identical digestive systems (Tattersall, 1993). This explains the moderate effect of species on the intestinal microbiota composition we observed in our study.

SEX AND AGE

We did not find any evidence for an influence of sex or age on the microbiota composition. It should be noted, however, that in this study different age-classes were not equally represented in the datasets, as we mostly sampled adult individuals (74.6% of all samples). Furthermore, all of the non-adults were at or beyond juvenile stage. Based on knowledge of microbiota development in human infants, the transformation of microbiota to an adult-like, mature composition occurs before reaching the juvenile stage (Koenig et al., 2011; Wopereis et al., 2014). Furthermore, we did not find any differences in alpha- and beta diversities of microbiota between males and females. Many studies showed an influence of sex in different species (Bolnick et al., 2014), including lemurs (Aivelo et al., 2016). However, as we pointed out before, other factors, such as occupancy, can outweigh the influence of this factor (Kovacs et al., 2011). Hence, we suggest that in the present study, the effect of sex on the faecal microbiota of the different *Eulemur* species might have been obscured by more influential factors, as well as relatively large variation in microbial composition between individual animals.

CONCLUSIONS

In conclusion, we showed that the intestinal microbiota in three genetically close species of lemurs was most strongly influenced by their occupancy, whereas the differences between species was minor, and influence of sex and age was not detectable. All three lemur species had a similar bacterial composition in terms of predominant and prevalent bacterial taxa. The findings reported here contribute to the knowledge base for the intestinal microbiota in non-human primates and factors that shape the bacterial composition in wild lemur populations, which can be extrapolated into general rules of intestinal microbiota assembly. Furthermore, the high fraction of poorly assigned taxa reinforces the notion that microbiota of non-humanoid primates has received little attention, harbouring a broad range of potentially novel bacterial species and genera that deserve attention in future studies.

CHAPTER 5

EFFECTS OF SEASONALITY AND PREVIOUS LOGGING ON FAECAL HELMINTH-MICROBIOTA ASSOCIATIONS IN WILD LEMURS

de Winter, I.I., Umanets, A., Gort, G., Nieuwland, W., van Hooft, W.F., Heitkönig, I.M.A., Kappeler, P.M., Prins, H.H.T., Smidt, H.

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ABSTRACT

Gastrointestinal helminth-microbiota associations are shaped by various ecological processes. However, the effect of the ecological context of the host in terms of geographic location, seasonality (i.e., dry versus wet season), and anthropogenic effects (i.e., logging history) on both groups of gastrointestinal inhabitants is unknown. We provide a first exploration thereof, and also examine the interactive effects between gastrointestinal helminths and microbiota. Fresh faecal samples (*N* = 335) from eight wild *Eulemur* populations were collected over a 2-year period across Madagascar. We used 16S ribosomal RNA gene sequencing to characterise the bacterial microbiota composition, and faecal flotation to isolate and morphologically identify nematode eggs. Infections with nematodes of the genera *Callistoura* and *Lemuricola* occurred in all lemur populations. Seasonality significantly contributed to the observed variation in microbiota composition, especially in the dry deciduous forest. Microbial richness and *Lemuricola* spp. infection prevalence were highest in a previously intensely logged site, while infections with *Callistoura* spp. showed no such pattern. In addition, we observed significant correlations between gastrointestinal parasites and bacterial microbiota composition in these lemurs. With this study we show that environmental conditions influence gastrointestinal nematodes and bacterial interactions in ways that, as far as we know, have not previously been reported.

INTRODUCTION

The gastrointestinal (GI) microbiota play an important role in the physiology, health, and nutrition of its host (Pfeiffer and Virgin, 2016). In addition, the GI microbiota can prevent gut colonisation by pathogenic microorganisms (Kabat et al., 2014). A stable and diverse GI microbiota composition was shown to be crucial for mammalian health (de Vos and de Vos, 2012; Sekirov et al., 2010), and defining the mechanisms influencing its composition and diversity is considered important (Patterson et al., 2014). Next to the microbiota, GI macroparasites, including protozoa and nematodes, can be present within a host's digestive tract. They can spread through the faecal-oral route, which involves ingestion of contaminated soil or food (Nunn et al., 2011). Parasitism can impact the host's health, behaviour, and survival, thereby influencing evolutionary processes and population dynamics (Ramanan et al., 2016). In addition, parasites are known to affect the host's reproduction directly through pathologic effects and mate choice, as well as indirectly by impaired nutrition and energy deficits (Leclaire and Faulkner, 2014).

Faecal bacterial GI microbiota and macroparasites living in internal body surfaces are part of an animals' microbiome and are involved in key host functions (Bennett et al., 2016). As studying wild populations under natural conditions is rather complex, most studies on the determinants of the GI microbiota composition and parasite prevalence either comprise laboratory or clinical studies that focus on a single host species or infection with a single parasite species (Eckburg et al., 2015; Tompkins et al., 2011). While these studies have provided important insights, understanding of ecological processes that shape composition and functionality of GI microbiota and parasites in wild populations is limited (Tompkins et al., 2011).

The composition of the GI microbiota is known to be shaped by multiple factors, including host genetics, evolutionary history, physiology, sex, and age (Barelli et al., 2015; Hansen et al., 2010). Several recent studies showed that the microbial composition can remain stable over the host's lifespan (Lozupone et al., 2012; McKenney et al., 2015). However, other studies found that extrinsic factors, including diet composition (De Filippo et al., 2010; Muegge et al., 2012; Wu et al., 2011), pathogens (Boutin et al., 2013), seasonality (Maurice et al., 2015), habitat degradation (Amato et al., 2013), and geographical differences (de Winter et al., 2018a; Dishaw et al., 2014) influence GI microbiota. For example, it has been shown that the microbial composition in black howler monkeys (*Alouatta pigra*) differs across seasons and is correlated with diet (Amato et al., 2015). Also, the distribution of parasite infections in wild host populations is influenced by a number of factors, including host susceptibility and exposure (Moore and Wilson, 2002). The nematodes that are the focus of the present study, spend part of their life cycle outside the host and are therefore exposed to environmental conditions that shape temporal variations in

parasite infections. Climatic seasonality has been identified as an important driver of this temporal variation in several wild primate species (Benavides et al., 2012; Huffman et al., 1997). However, studies investigating these links have yielded different outcomes (Aivelo et al., 2016; Amato et al., 2015; Barrett et al., 2013). It has also been shown that some nematodes have an accelerated development and increased reproduction and survival rates in wetter and warmer conditions (Benavides et al., 2012; Nunn and Altizer, 2006), and desiccate more frequently under dry circumstances (Huffman et al., 1997). Several studies found GI parasite richness, prevalence, and abundance to be higher in the warm wet season, compared to the cold dry season, e.g., in lemurs (Huffman and Chapman, 2009; Raharivololona and Ganzhorn, 2010; Setchell et al., 2007), chimpanzees (Huffman et al., 1997), howler monkeys, and spider monkeys (*Ateles geoffroyi*) (Maldonado-López et al., 2014). However, some helminth species (e.g., *Enterobius* spp.) seem to prefer relatively low temperatures (Caldwell, 1986). Although the underlying processes remain unclear (Hanson et al., 2012), these examples show that environmental factors are able to influence the microbial composition and parasite prevalence (Barelli et al., 2015; Maurice et al., 2015), and require further study in wild mammals.

In addition to environmental factors, the impact of anthropogenic forest disturbance, such as logging, on health and pathogens in both wildlife and humans may be far reaching (Keele et al., 2006). Anthropogenic forest disturbance may lead to changes in host population densities and interaction patterns of wildlife with humans, domestic animals, and other wildlife species (Gillespie et al., 2005; Nunn and Altizer, 2006). Such disturbances can thereby enforce changes in the GI microbiota composition and parasite infections (Amato et al., 2013; Barelli et al., 2015; Chapman et al., 2005). Microbiota diversity can be reduced in degraded areas, as is shown in howler monkeys, red colobus monkeys (*Procolobus gordonorum*), and other primate species (Amato et al., 2013; Barelli et al., 2015; McCord et al., 2014). Furthermore, increased parasite prevalence, virulence, and transmission rates were found in such disturbed forests (Chapman et al., 2006; Chapman et al., 2005; Kowalewski et al., 2011). Although the exact mechanisms influencing the microbial composition and parasite infections in disturbed forests is still unknown, nutritional stress is considered important (Chapman et al., 2006). Nutritional stress can alter the microbiome and lower an animal's immune status, resulting in a higher susceptibility to parasites (Hughes and Kelly, 2006). Forest disturbance can also directly influence parasites that spend part of their life cycle outside of the host, as changes in forest structure lead to differences in light exposure, temperature, and humidity (Angelstam et al., 2004). Despite the relevance of understanding parasite and microbiome ecology in wild primates living in natural versus human-modified forests, an integrated study on forest disturbance effects on both parasites and the microbiome has, as far as we know, not

been performed before.

Microbiota and parasites co-inhabit the GI-tract and have evolved in close association, suggesting that they have the potential to influence each other (Kreisinger et al., 2015). Research on this interplay between host, parasites, and the microbiome has increased over the last decade (Mutapi, 2015) and recent studies in humans showed associations between nematode infections and changes in the GI microbiota structure (Kay et al., 2015; Lee et al., 2014; Morton et al., 2015). However, this observation is not consistent across human populations (Cantacessi et al., 2014; Cooper et al., 2013). Another study experimentally demonstrated that the gut bacterial composition in mice (*Mus musculus*) can change when exposed to a GI parasite (*Trichuris muris*) (Houlden et al., 2015). Associations between specific bacteria and the abundance of enteric nematodes were also found in wild wood mice (*Apodemus sylvaticus*) (Maurice et al., 2015). Most of these aforementioned studies focussed on mice, pigs (*Sus scrofa*), or humans. However, recent studies have begun to address the interaction between the microbiome and parasites in primates (McKenney et al., 2017). We aim to contribute with this study by providing comparative data on the interactive effect of parasite infections and microbiota composition of wild lemurs.

Specifically, we aim to assess the effects of seasonality (i.e., dry versus wet season), and forest disturbance (i.e., high and low levels of logging) on the interaction between GI parasites and bacterial microbiota composition in two lemur species. Recently, the microbial composition of lemurs has been studied in captive lemurs (McKenney et al., 2015), in two sympatric wild lemur species (Fogel, 2015), and in wild sifakas (Springer et al., 2017). However, the processes leading to the natural variation of faecal microbiota in wild lemurs, and how this variation is influenced by environmental conditions, need further study. Furthermore, only a few studies to date have used a metataxonomic 16S ribosomal RNA (rRNA) gene-targeted approach to address the association and interactive effects between parasites and the microbiome (Cantacessi et al., 2014; Cooper et al., 2013; Houlden et al., 2015; Kreisinger et al., 2015; Lee et al., 2014; Li et al., 2012; Rausch et al., 2013; Walk et al., 2010). In the present study, we focus on four congeneric lemur species at eight geographic locations: *Eulemur rufifrons*, *E. fulvus*, *E. macaco*, and *E. rubriventer*. The large heterogeneity in lemur habitats across Madagascar is created by an interaction of the east-west and north-south rainfall gradient (Irwin et al., 2005). The four lemur species belong to the genus *Eulemur* and are morphologically alike (Markolf et al., 2013), are present in the distinct geographic regions of Madagascar, and inhabit both large intact forests and forest that have experienced past logging (Wright et al., 2012).

Given the major role of environmental factors in shaping seasonal variation in microbial community structure and parasite infections, we expected that (1) lemurs

inhabiting the dry deciduous forests of western Madagascar with strong seasonal variation in rainfall and temperature show larger seasonal contrasts in both parasite infections and microbial composition compared to lemurs in the rainforests of eastern Madagascar with less seasonal variation. We further expected (2) that the microbiota composition is altered and parasite infection prevalence is increased in lemurs whose habitat is restricted to previously logged rainforests compared to lemurs living in less disturbed forests. Lastly, we explored (3) correlations between GI microbiota and natural parasite infections. In this study we determine how the wild lemur GI microbiota and parasite infections vary with their geographic distribution, with seasonal variation, and with past logging. In addition, we explore the interactive effects between the parasites and microbiota present.

METHODS

STUDY SITE

Our research was performed in eight geographically distinct sites (Fig. 5.1, Table 5.1). Kirindy Forest, Ankarafantsika National Park (NP), and Zombitse NP are located on the western, north-western, and south-western side of Madagascar, respectively. They consist of dry deciduous forest with pronounced seasonality (Goodman et al., 2005). These western regions have a higher annual mean temperature than the eastern rainforests, but receive less rainfall.

In contrast, Andasibe Mantadia NP (including Mitsinjo) and Ranomafana NP are located on the eastern side of Madagascar and are relatively wet rain forests with a less distinct dry season compared to the western areas (Irwin et al. 2010). Within Ranomafana NP, we distinguished two research sites, Talatakely (TALA) and Vatoharanana-Valohoaka (VATO-VALO), with different degrees of anthropogenic disturbance (Fig. 5.2) (Balko and Underwood, 2005; de Winter et al., 2018a). Before the establishment of the national park in 1991, the forests in this area were used by local inhabitants for slash-and-burn agriculture, amongst others (Irwin et al., 2010). Now, more than 25 years after the last logging activities, Ranomafana NP shows a high heterogeneity in forest structure (de Winter et al., 2018a).

The islands Nosy Be, Nosy Komba, and Nosy Tanikely are located in the north-west of Madagascar. The forests of Nosy Be ($\sim$320 km^2) are largely replaced by coffee, fruit, and ylang-ylang plantations, and by rice and sugar cane fields. Only Lokobe NP ($\sim$7 km^2) on the south-eastern part of the island still contains the island's original forest vegetation. Nosy Komba and Nosy Tanikely are located in between Nosy Be and the mainland. The vegetation on Nosy Komba ($\sim$25 km^2) is similar to Nosy Be. The vegetation on Nosy Tanikely ($\sim$0.06 km^2) mainly consists of low forest and bushy vegetation, including palm trees and planted banana and mango trees, surrounded by

a sandy shore with large rock formations (I. de Winter, *personal observation*, Köhler et al. 1998).

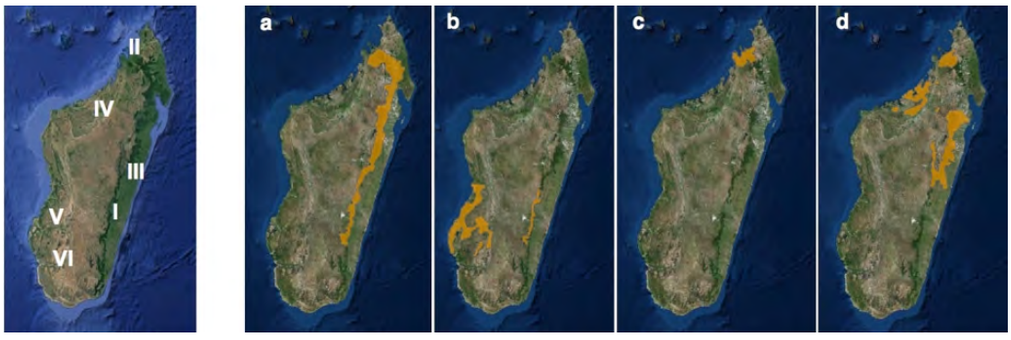

Figure 5.1: Study sites and the geographic ranges of the different Eulemur species. (Google Maps, 2015). Left: map of Madagascar with the study sites Ranomafana NP (I); Nosy Be, Nosy Komba, and Nosy Tanikely (II); Andasibe NP (III); Ankarafantsika NP (IV); Kirindy Forest (V); and Zombitse NP (VI). Right: the geographic ranges of a) *Eulemur rubriventer*; b) *E. rufifrons*; c) *E. macaco*; d) *E. fulvus* (IUCN, 2016). Downloaded from The IUCN Red List of Threatened Species. Version 2016-3. www.iucnredlist.org on 12 February 2017.

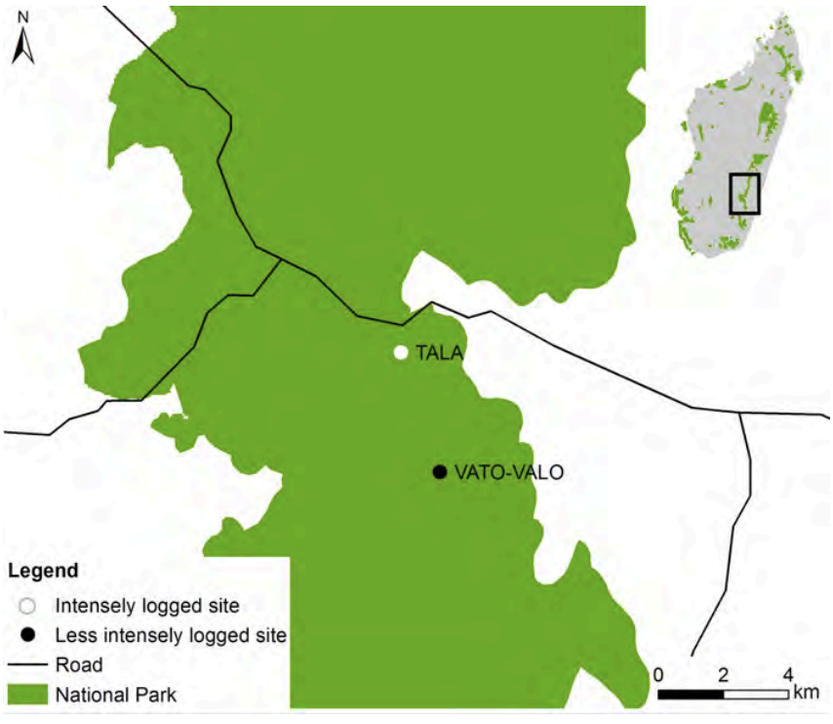

Figure 5.2: Map of Ranomafana National Park and the two forest sites that were surveyed in this study. Talatakely (white dot) experienced relatively intense logging in the past, while Vatoharanana-Valohoaka (black dot) experienced no such disturbances. This map was generated via ArcGIS version 10.5. Data was downloaded from UNEP-WCMC and IUCN (2016), Protected Planet: National Parks of Madagascar; The World Database on Protected Areas (WDPA) (Online), May 2016, Cambridge, UK: UNEP-WCMC and IUCN.

Table 5.1: Lemur (*Eulemur* spp.) study sites in Madagascar.

Study site	GPS coordinates (S, E)	Area (km^2)	Annual rainfall (mm)	Mean temperature (annual range, °C)	Altitude (range, m)	Lemur species	Sample (*N*)
Ranomafana NP	21.27, 47.33	435	3000	11 – 25	500 – 1500	*E. rufifrons* *E. rubriventer*	48 68
Nosy Be	13.33, 47.25	252	2250	15 – 35	0 – 430	*E. macaco*	18
Nosy Komba	13.47, 48.35	25	2250	15 – 35	0 – 620	*E. macaco*	23
Nosy Tanikely	13.47, 48.23	0.3	2250	15 – 35	0 – 47	*E. fulvus*	17
Andasibe NP	18.92, 48.42	155	1680	10 – 27	900 – 1060	*E. fulvus*	43
Ankarafantsika NP	16.25, 46.80	1350	1300	17 – 28	80 – 330	*E. fulvus*	50
Kirindy Forest	20.07, 44.67	722	767	19 – 31	20 – 90	*E. rufifrons*	40
Zombitse NP	22.87, 44.68	200	740	14 – 30	485 – 825	*E. rufifrons*	31

STUDY SPECIES

True lemurs (genus *Eulemur*, family Lemuridae) are medium-sized (body and tail length 30 to 50 cm, 2 to 4 kg) arboreal primates that occasionally move quadrupedally on the ground. They live in social groups ranging from two to fifteen individuals and their diet primarily consists of fruits, flowers, and leaves (Markolf et al., 2013). We studied four *Eulemur* species: *Eulemur rufifrons, E. fulvus, E. macaco*, and *E. rubriventer. Eulemur rufifrons* lives in the south-west and east and the native range of *Eulemur fulvus* is in the north of Madagascar, on both the east and west side (Mittermeier et al., 2008). *Eulemur fulvus* has also been introduced to the northern island Nosy Tanikely. *Eulemur macaco* is found on the mainland and several islands in the north-west, while *Eulemur rubriventer* inhabits forests in eastern Madagascar (Fig. 5.1, Table 5.1). *Eulemur rubriventer* and *E. rufifrons* live sympatrically in Ranomafana NP (Overdorff, 1993).

FAECAL SAMPLE COLLECTION

We collected 338 faecal samples between October 2013 and February 2015 (Table 5.1), of which 103 in Ranomafana NP. Here, we collected 38 samples from a previously logged site (Talatakely) and 65 from a less disturbed site in terms of its logging history (Vatoharanana-Valohoaka). Immediately after defecation, fresh faecal samples (3 to 4 g) were collected from the forest floor, non-invasively. We noted visual characteristics, i.e., consistency, colour, presence of blood, mucus, or tapeworm proglottids. We also reported GPS coordinates, time, group size, group composition, age (sub-adult if < 2 years old or adult if > 3 years old), and sex. We allocated a body fur condition score to the individuals whose faeces were collected (Berg et al., 2009). We aimed at sampling all adults within a social group and did not resample individuals. As soon as we were no longer sure whether faeces was from a new individual or whether we already sampled the animal, we moved to another group. As we worked mostly within national parks or reserves, the lemurs were all habituated to human observers, mainly due to the frequent visits by tourists or researchers, which facilitated the faecal collection. We found no abnormalities in the consistency and colour of the faeces and we did not find blood, mucus, or tapeworm proglottids in any of the faecal samples. Within twelve hours after collection, each faecal sample was divided over two sterile tubes: 1 g of faeces was stored in a tube filled with 5 ml of 70% ethanol and 2 g of faeces was placed in a tube filled with 15 ml SAF fixative (Chapman et al., 2006; Van Gool et al., 2003). Samples were analysed at the Laboratory of Microbiology, Wageningen University, and the Department of Infectious Diseases and Immunology, Utrecht University. All described methods were performed in accordance with the relevant guidelines and regulations and was approved by the trilateral commission (CAFF/CORE) in Madagascar (permits 297/13 and 143/14/MEF/SG/DGF/DCB.SAP/SCBSE).

DNA-BASED BACTERIAL COMPOSITION ANALYSES

Faecal bacterial microbiota composition, determined by next generation sequencing of 16S rRNA gene fragments, was used as proxy for the intestinal microbial community. We extracted microbial DNA from the faecal samples collected in Ranomafana NP, following a modified double bead-beating procedure using the QIAamp® DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) (Based on Yu and Morrison 2004). For the sample processing, we used the protocol proposed by Yu & Morrison (2004) and modified by Salonen et al. (2010). Prior to DNA extraction, faecal material was air-dried during 15 to 20 min in a fume hood to remove ethanol from the samples. We extracted DNA from samples collected at the other sites using the Maxwell® 16 Research Instrument (Promega, Madison, USA) in combination with the corresponding RNA extraction kit customised for faecal DNA extraction according to manufacturer's instructions. Prior to DNA extraction, samples were rehydrated through series of ethanol solutions with decreasing proportions of ethanol in steps of 10%. For rehydration, 1.5 ml of 70% ethanol with faecal particles was transferred into a fresh 2 ml tube and centrifuged at 13,000 rpm for 5 min. After centrifugation, part of the supernatant was replaced with the same amount of distilled water to decrease ethanol concentration by 10 percentage points, vortexed, and incubated for 10 min at room temperature. These steps were repeated until the ethanol was completely replaced by distilled water. Cell disruption and lysis was performed as described above, but instead of lysis buffer we used S.T.A.R. buffer (Roche Molecular Systems, USA). DNA quality and concentration were spectrophotometrically verified (Nanodrop Technologies, Wilmington, USA). For each sample, barcoded amplicons were amplified from 40 ng of extracted DNA using a two-step PCR method in a LabCycler Gradient (SensoQuest, Germany) and pooled afterwards as described previously (Tian et al., 2016). Briefly, the V1 - V2 region of the 16S rRNA was first amplified by PCR (25 cycles of 95°C (30 s), 52°C (40 s), and 72°C (90 s)), followed by post-elongation (72°C, 7 min) using primer pair 27F–DegS: 5'–GTTYGATYMTGGCTCAG–3' (van den Bogert et al., 2011) and 338R–I: 5'–GCWGCCTCCCGTAGGAGT–3' / 338R–II: 5'–GCWGCCACCCGTAGGTGT–3' (Daims et al., 1999) that contained forward and reverse linkers UniTag I (5'–GAGCCGTAGCCAGTCTGC–3') and UniTag2 (5'–GCCGTGACCGTGACATCG–3'), respectively. Amplicons were then used as template for a second PCR in order to introduce sample-specific barcodes, using individual barcode primers targeting Unitag1 and UniTag2 sequences. The amount and size of the amplicons were checked visually by agarose gel electrophoresis. The PCR products were purified using the HighPrepTM PCR kit (MagBio Genomics), concentrated using magnetic beads (MagBio, Switzerland) according to the HighPrep protocol, quantified using the Qubit dsDNA BR Assay Kit (Life Technologies, USA), and pooled in equimolar amounts into libraries of 48 samples, including two mock

communities of defined composition, for paired-end sequencing (300 bp) on the Illumina Miseq platform at the European Genome and Diagnostics Centre (GATC Biotech, Constance, Germany). Mock communities, i.e. mixes of quantified and purified copies of bacterial 16s rRNA genes in known proportions, are routinely used in the laboratory to assess quality and reliability of a sequencing run, amplicon preparations, and quality of data processing, as was described previously (Ramiro-Garcia et al., 2016). The amplicon sequences were demultiplexed and the subsequent analysis of raw rRNA gene sequence data was performed using NG-Tax (Ramiro-Garcia et al., 2016). Reads assigned to OTUs of plant origin such as chloroplast and plant mitochondrial DNA were removed from the dataset used for downstream analysis. The raw data was ranked per individual sample based on the matching of reads to OTUs, allowing an error of one nucleotide.

PARASITE ISOLATION

The collected faecal samples were examined for the presence of GI nematodes with the use of the Centrifugation-Sedimentation-Flotation (CSF) method (Dryden et al., 2005). GI nematode species identification was based on morphological traits such as colour, shape, size, and content of eggs (Clough, 2010; Gillespie, 2006; Irwin and Raharison, 2009). A rough estimation of the number of parasite eggs per gram of faeces (EGP) was obtained by simple counts. Since the number of eggs that end up in the faeces is not a reliable index of adult worm burden (Gillespie and Chapman, 2006), the egg count cannot be regarded as a measurement of infection intensity, but rather as a measurement of infectivity.

STATISTICAL ANALYSIS

After initial sequence data processing with NG-tax, we combined the OTU table, metadata, and phylogenetic tree into a 'phyloseq' object, as implemented in the 'phyloseq' R package (v.1.22.3). Further analyses were carried out in R (v 3.4.1). OTUs that were encountered in less than three samples, OTUs not assigned to any taxonomic level (NA), and OTUs identified as chloroplast and mitochondria were removed. In addition, samples with a low number of reads (less than 1000 reads), missing metadata of interest, and one sample (i.e., 'NT9F', due to the low quality of the starting material) were removed from the data set. For beta diversity analysis, the weighted UniFrac distance matrix was calculated from the OTU table and phylogenetic tree as implemented in the 'phyloseq' package, with phylogenetic tree rooted at midpoint (package 'phangorn'). Multidimensional scaling with weighted UniFrac as a distance matrix (PCoA) was applied (package 'phyloseq') to obtain a first insight into the beta diversity of faecal microbial communities in the investigated lemur populations. We used dbRDA to identify explanatory variables that significantly

contributed to explaining the observed variation in microbial composition (package 'vegan'). Variable 'Social Group' was excluded from the analysis due to extremely uneven sample distribution, with 28 out of a total of 92 social groups including only one sample. The degree to which individual factors could explain microbiota composition was estimated by partial dbRDA with control for variables that were not used as a constraint, but identified as significant by stepwise dbRDA. Significance of the grouping was estimated by one-way analysis of variance (ANOVA), and R^2 values were used as estimator of variation explained by a constraint (package 'vegan'). Phylogenetic diversity was used as a primary alpha diversity measure and was calculated from the phyloseq object with the OTU table rarefied at a read depth of 1051, using a custom function (author T.W. Battaglia). Statistical differences between alpha diversity of pre-defined sample groups was assessed by posthoc Kruskal Nemenyi-tests (package 'PMCMR'). The datasets generated during this study are available in the public read archive EBI, named 'ena-STUDY-WAGENINGEN UNIVERSIT-03-04-2017-14:57', with accession number 'PRJEB20227'.

To analyse the effect of seasonality (early dry vs early wet season) and location (western dry deciduous forests vs eastern rainforests) on the infection prevalence of *Callistoura* and *Lemuricola* spp. in *Eulemur* species, GLMMs were used, assuming a Bernoulli distribution and logit link function. We included random effects for sites within location and for social group within sites into the model and fixed effects for season, location, and their interaction. We focused specifically on the interaction between location and season in order to test the seasonality hypothesis as formulated in the introduction. Three covariates at the individual lemur level (sex, age, and body fur score) were available for 263 of the 335 individuals. The covariates were introduced into the model, but were removed if found to be unimportant ($P > 0.05$), as otherwise 72 observations with missing covariate values would be lost for analysis. Random effects remained in the models, as they reflect the data collection procedure. To present estimated infection prevalence with 95% confidence intervals on the probability scale, we back-transformed the results (on the logit-scale) from the GLMMs first and then applied a shrinkage factor (Zeger et al., 1988), which is needed for GLMMs to obtain predicted population means instead of medians. To test whether infections by the two nematode genera occurred independently, we modified the GLMM for *Callistoura* spp. by adding an indicator variable for *Lemuricola* spp. as regressor to the model. In this way, we allowed the infection prevalence for *Callistoura* spp. to be different for the lemurs infected with *Lemuricola* spp. or uninfected animals.

We analysed *Callistoura* and *Lemuricola* spp. infection prevalence in disturbed versus less disturbed sites using a subset of the data (Ranomafana NP; $N = 103$). Here, we aggregated infection scores per social group and used ordinary Generalised Linear Models (GLMs) assuming a binomial distribution for the number of infected

animals per group and logit link function. We entered main effects for the factors disturbance (less vs more disturbed), season (early dry vs early wet), and species (*E. rubriventer* vs *E. rufifrons*) into the model. In the analysis of *Callistoura* spp. prevalence, we also included two-way interactions, but this was not possible for *Lemuricola* spp. prevalence due to low numbers of cases (fourteen cases, with just one in the less disturbed site). Extra-binomial variation could not be ruled out, because individuals within social groups may have correlated responses. Because of different group sizes (range one to seven), we used Williams' method as available in the 'dispmod' package of R (Scrucca, 2012). If the overdispersion parameter was estimated to be zero, we used an ordinary binomial GLM. We calculated back-transformed predicted means presented with 95% confidence intervals for the previously disturbed and less disturbed sites.

The statistical analyses were performed using base R (R Core Team, 2017) , the R packages 'lme4' for the GLMMs (Bates et al., 2015), and 'lsmeans' (Lenth, 2016) for prediction of group means. Hypotheses were tested using Likelihood Ratio Tests (LRT), comparing the log-likelihoods of the full model with the model without the effect of interest. For the GLM analysis with extra-binomial variation, LRT and Wald-tests were used. We took a full model approach, in which we first tested for all fixed effects simultaneously. Regardless of the result from this omnibus test, we tested the specifically formulated hypotheses regarding seasonality and forest disturbance and main effects were tested in presence of interactions.

RESULTS

SEASONALITY

We found clear separation of samples by season in the bacterial microbiota composition of multiple lemur populations sampled across Madagascar, using principal coordinate analysis (PCoA) based on the weighted UniFrac distance matrix (early wet season $N = 92$, early dry season $N = 133$, $R^2 = 0.11$, Adonis; $P = 0.001$, Fig. 5.3). In the two-directional stepwise distance-based redundancy analysis (dbRDA), area of sample collection was identified as the most influential variable, followed by season, when considering all samples. We observed an increase in the percentage of explained variance in microbiota composition by seasonality when focussing on samples collected within one area and one lemur species. Specifically, for *E. fulvus* populations from Ankarafantsika NP and Andasibe NP, and *E. rubriventer* and *E. rufifrons* populations from Ranomafana NP, the percentage of variation in microbiota composition explained by season increased from 5.7% for the entire dataset to 16.9%, 20.2%, 12.5%, and 13.5%, respectively (Fig. 5.4a-d). Therefore, these populations harboured a different microbial composition in the early dry season compared to the

early wet season. With regards to alpha diversity, the *E. fulvus* population in Ankarafantsika NP showed a significantly higher mean phylogenetic diversity (PD index, $P = 0.0002$) in the early dry season ($N = 21$) compared to the early wet season ($N = 29$). No statistically significant differences in alpha diversity were observed for other subsets of samples as defined by area of habitation and lemur species.

Based on morphological analyses, nematode species of two genera, *Callistoura* and *Lemuricola* (Fig. 5.5), were present in the GI tract of nearly all *Eulemur* individuals from eight geographically distinct populations. Of all the sampled lemurs ($N = 335$), 188 (56.1%) were only infected with *Callistoura* spp., 17 (5.1%) were only infected with *Lemuricola* spp., 34 (10.1%) were infected with both nematode species, and 96 (28.7%) were not infected (Table 5.2). The observed co-occurrence (10.1%) is very close to the expected co-occurrence for independent infections (67.5% x 15.1% = 9.9%), suggesting that infections with both *Callistoura* and *Lemuricola* spp. occur independently, and therefore, co-infection appears to be independent.

In the generalised linear mixed model (GLMM) on *Callistoura* prevalence across different seasons, we did not find effects of the covariates age, sex, and body fur score (1-df Likelihood Ratio Tests (LRT); all $P > 0.05$). These covariates were therefore removed from the model, and the resulting model comprised fixed effects for season, location and their interaction, and random effects for sites within location and social groups within sites. We found significant variation between sampling areas (variance component = 1.50; $P < 0.001$; LRT) and social groups (variance component 1.12; $P < 0.001$; LRT). We found no effect of season, location, and their interaction (3-df LRT; $P = 0.61$), rejecting our hypothesis that the seasonal difference in infection prevalence is lower in the eastern rainforests than in the western dry deciduous forests. Estimated *Callistoura* spp. prevalence in the eastern areas was 64% in the early dry season and 66% in the early wet season. In lemur populations from western forests, the infection prevalence was 60% in the early dry season and 75% in the early wet season. The seasonal contrast did not differ significantly between the western and eastern area (LRT; $P = 0.345$ for interaction between season and location).

In the seasonality GLMM on *Lemuricola* spp. prevalence, no effects of covariates were found (all $P > 0.05$; LRT). We found significant variation between social groups (variance component 0.80; $P = 0.02$; LRT), but not between sampling areas (variance component 0.0; $P = 0.50$). No effect of season, location, and their interaction were found (3-df LRT; $P = 0.34$). The seasonal contrast did not differ between the western and eastern areas (LRT; $P = 0.84$ for interaction season x location). In addition, we found no difference in infection prevalence of *Callistoura* spp. between animals with and without *Lemuricola* spp. infection (LRT; $P = 0.41$). Overall, we found that 188 out of 284 lemurs without *Lemuricola* spp. were infected with *Callistoura* spp. (66%), and 34 out of 51 *Lemuricola* spp. infected animals were infected with *Callistoura* spp. (67%).

Table 5.2: *Callistoura* and *Lemuricola* spp. prevalence (0-1) in lemur populations at different areas across Madagascar.

	No nematodes	*Lemuricola* spp.	*Callistoura* spp.	*Callistoura* and *Lemuricola* spp.	Total (*N*)
Andasibe NP	0.37	0.02	0.56	0.05	43
Ankarafantsika NP	0.7	0.06	0.2	0.04	50
Kirindy Forest	0.05	0.05	0.73	0.16	37
Nosy Be	0.22	0.11	0.61	0.06	18
Nosy Komba	0.13	0.13	0.48	0.26	23
Nosy Tanikely	0.29	0.06	0.53	0.12	17
Ranomafana NP	0.25	0.03	0.62	0.1	116
Zombitse NP	0.07	0.03	0.77	0.13	31
Total	**0.29**	**0.05**	**0.56**	**0.1**	**335**

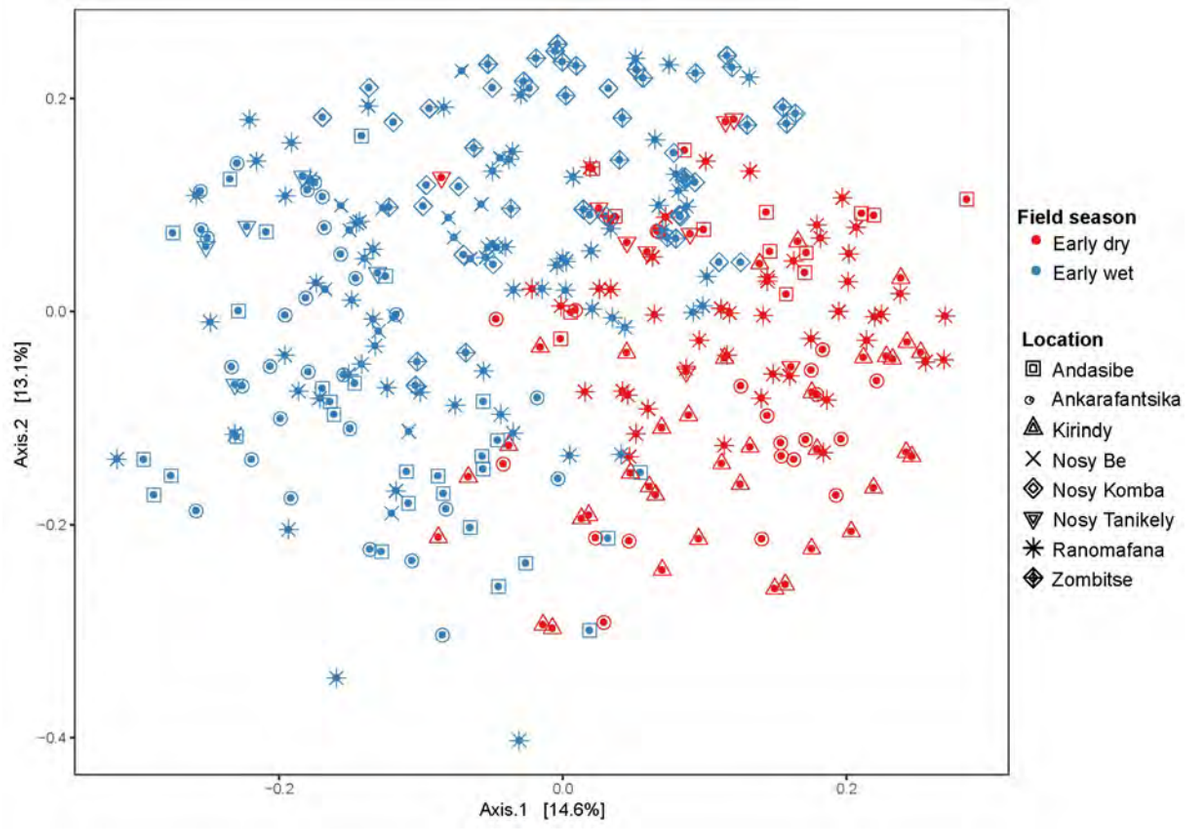

Figure 5.3: Lemur faecal microbiota composition across seasons and locations. Ordination of faecal microbial composition in multiple lemur populations across Madagascar sampled in different seasons (early dry and early wet) and locations. This figure shows the results of a principal coordinate analysis (PCoA) based on the weighted UniFrac distance matrix, grouping strength of samples by season R^2 = 0.09 (Adonis; P = 0.001).

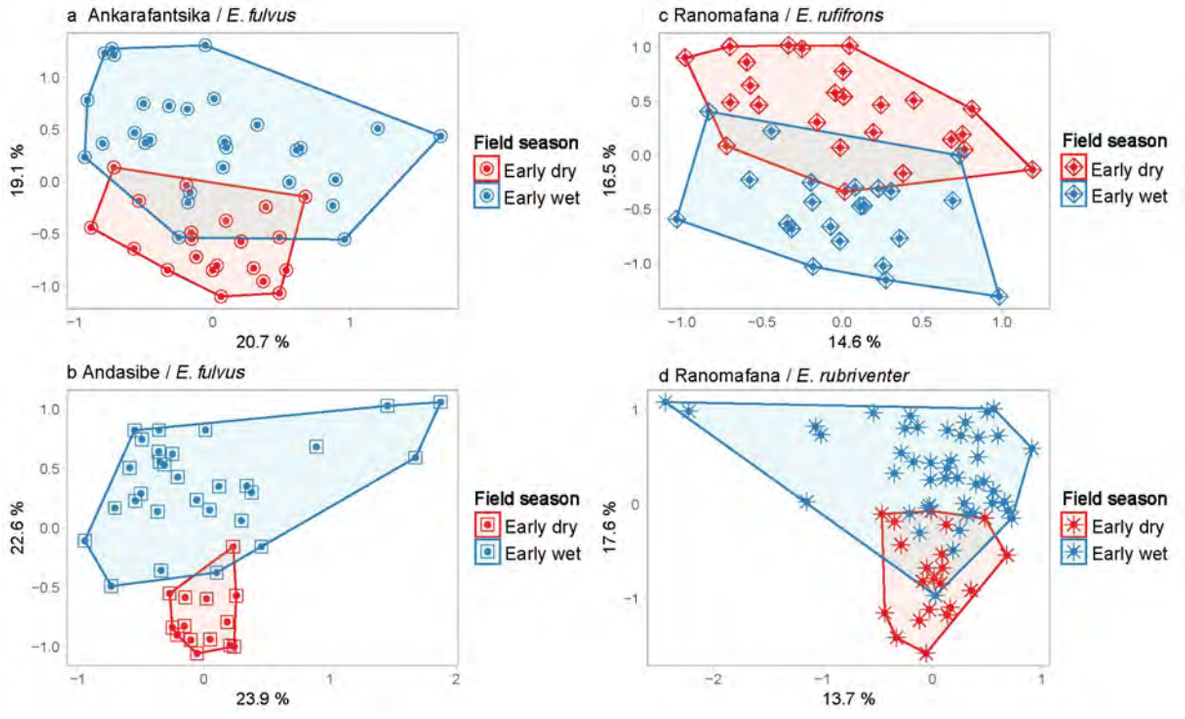

Figure 5.4: Lemur faecal microbiota composition across seasons and locations. dbRDA Analyses of the abundance-weighted phylogenetic composition at OTU level of individual lemurs across seasons (early dry and early wet) in different geographic areas visualised in ordination. Faecal microbiota significantly cluster by season. Results are given for the percentage of variation explained by the sum of the first two canonical axes, percentage explained by season with corresponding *P*-value. a) *Eulemur fulvus* in Ankarafantsika National Park, (39.8%, 16.9%, $P = 0.001$); b) *Eulemur fulvus* in Andasibe (46.5%, 20.2%, $P = 0.001$); c) *Eulemur rufifrons* in Ranomafana NP (31.1%, 13.5%, $P = 0.001$); d) *Eulemur rubriventer* in Ranomafana NP (31.3%, 12.5%, $P = 0.001$).

Figure 5.5: Detected parasite species. *Callistoura* sp. egg (left) and *Lemuricola* sp. egg (right), isolated from a faecal sample of *Eulemur rufifrons*, magnification 200x (*pictures taken by I. de Winter*).

DISTURBANCE

A possible association between forest disturbance and parasite infection and faecal bacterial microbiota composition was examined in lemurs from Ranomafana NP. Bacterial richness was significantly higher in the previously logged site (Talatakely, *N* = 29), compared to the less disturbed site (Vatoharanana-Valohoaka *N* = 27, PD index= 7.3±1.1 vs 5.8±1.7, *P* = 0.001). The dbRDA also showed that the microbial composition was grouped according to sites with a different disturbance history (*P* = 0.004, Fig. 5.6).

In the analysis of prevalence of *Callistoura* spp. we did not find significant effects of season, species, location or their interactions (6 df LRT; *P* = 0.36). The prevalence of *Callistoura* spp. seemed to be higher in the less disturbed site compared to the previously logged site (back-transformed means: 83.0% vs 53.0% respectively; Wald test *P* = 0.084, Fig. 5.7a). In the analysis of prevalence of *Lemuricola* spp., the omnibus test showed a significant difference (3 df LRT; *P* < 0.001), with a lower prevalence in the less disturbed compared to the previously logged sites (1.2% vs 26.2% respectively, *P* < 0.001, Fig. 5.7b). The infection rates of *Callistoura* spp. showed considerable extrabinomial variation, but the infection rates of *Lemuricola* spp. did not show such a pattern.

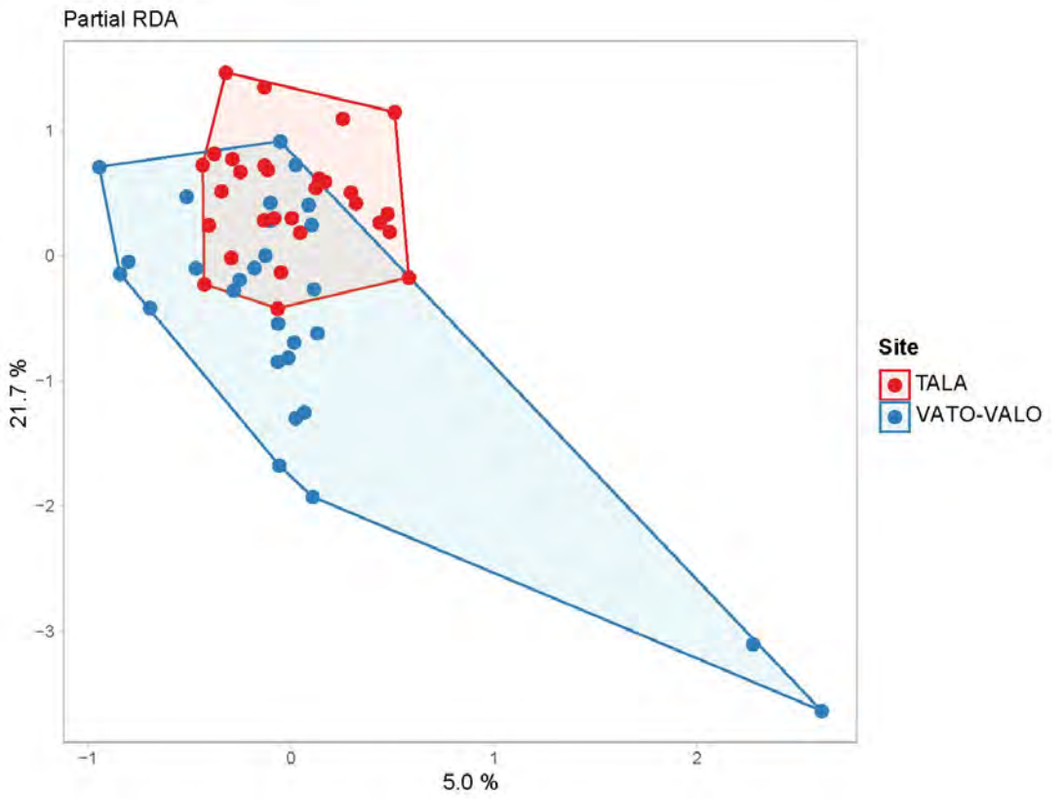

Figure 5.6: Faecal microbiota composition in disturbed and less disturbed sites. Ordination (RDA) of the microbial composition (OTU) across sites with a different disturbance history (disturbed vs less disturbed) for *Eulemur rubriventer* and *E. rufifrons* in Ranomafana National Park, Madagascar. Cumulative variation explained by the first two axes was 26.7% and the sampling location accounted for 3.8% of the total variation ($P = 0.002$).

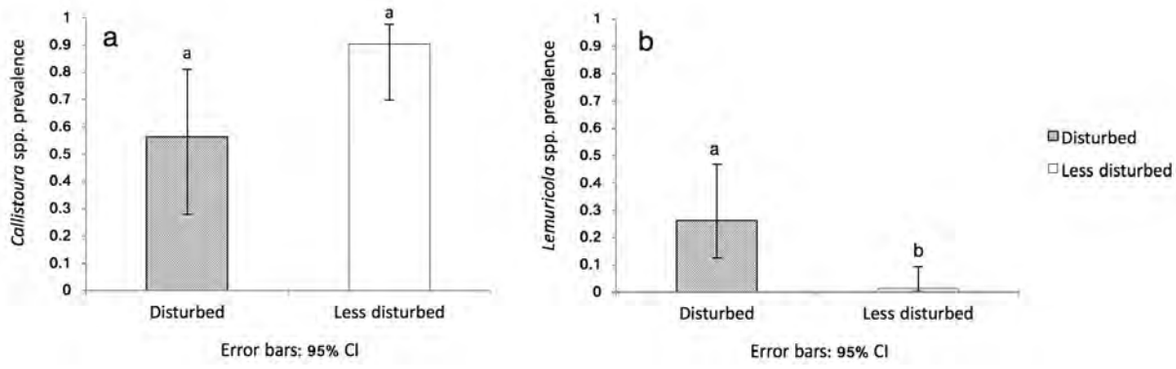

Figure 5.7: Parasite prevalence with disturbance. Prevalence of a) *Callistoura* spp. and b) *Lemuricola* spp. in *Eulemur rufifrons* and *E. rubriventer* populations in a previously disturbed and less disturbed site in Ranomafana NP, Madagascar. Mean with 95% confidence intervals and the letter coding above the bars indicate whether groups are significantly different.

MICROBIOTA AND PARASITES

Using bi-directional stepwise dbRDA, the variables site, species, field season, *Callistoura* spp. prevalence (CallPrev), sex, and age were identified to significantly contribute to explaining the observed variation in faecal microbial composition ($P < 0.05$), with explanatory power of 12.5%, 1.1%, 5.7%, 0.4%, 1%, and 0.8%, respectively. Our results revealed a small, but significant, correlation between microbiota composition and *Callistoura* spp., but not with *Lemuricola* spp. To this end, constrained ordination (partial dbRDA) showed that prevalence of *Callistoura* spp. accounted for 0.4% ($P = 0.025$) of the variation in microbiota composition found among all samples with available microbial and parasite infection data ($N = 324$), regardless of host species and habitation (de Winter et al., 2018b).

When focussing on lemurs of one species from the same area and season, we could not find statistically significant correlations with *Callistoura* spp. prevalence. However, among the *E. rubriventer* population in Ranomafana NP in the early dry season, microbiota composition showed significant correlation with *Lemuricola* spp. prevalence ($P = 0.046$) with 9.2% of variation explained by this factor. Interestingly, a clear separation of samples could be observed in the corresponding dbRDA plots, albeit without statistical support (all $P > 0.05$), probably due to the relatively low and unequal number of samples per group.

DISCUSSION

We assessed the influence of environmental conditions on the faecal bacterial microbiota composition and parasite infections as well as the correlation between GI microbiota and parasites in wild lemurs. The two helminth genera *Callistoura* (Chabaud and Petter, 1959) and *Lemuricola* (Chabaud et al., 1965) were detected in all *Eulemur* populations. These microphagous pinworms belong to the family Oxyuridae and are directly transmitted (Irwin and Raharison, 2009). They colonise distinct parts of the gut of their hosts: *Callistoura* spp. lives in the ileum and colon and *Lemuricola* spp. in the caecum and colon (Irwin and Raharison, 2009). These parasite species were also found in most other lemur genera at various locations (Chabaud et al., 1965; Irwin and Raharison, 2009), including other species from the genus *Eulemur*, i.e. in *E. flavifrons* (Schwitzer et al., 2010), *E. macaco* (Junge and Louis, 2007), *E. fulvus* (Nègre et al., 2006), and *E. albifrons* (Junge et al., 2008). Thus, these nematode genera have a very broad distribution throughout Madagascar and do not show obvious specificity to a particular kind of lemur host (Irwin and Raharison, 2009).

SEASONALITY

We hypothesised that lemurs inhabiting dry deciduous forests, with strong seasonal variation in rainfall and temperature, would show larger seasonal contrasts in both parasite infections and microbial composition compared to lemurs in eastern rainforests with relatively low seasonal variation. Nevertheless, we found a strong seasonal contrast in the microbial composition at Organisational Taxonomic Unit (OTU) level across all lemur populations. Across Madagascar, lemurs are exposed to seasonality and have been observed to change their diet accordingly (Wright et al., 2012). Diet was found to be an important driver of the GI microbial composition in many human studies (e.g., Filippo and Cavalieri 2010). Although humans are assumed to have a stable microbiota over longer periods of time (> 10 days) (Rajilić-Stojanović et al., 2013), dietary changes can alter the relative abundance of specific members of the microbiota within 24 hours (Wu et al., 2011). With respect to wildlife, for example wood mice (*Apodemus sylvaticus*) were shown to exhibit seasonal shifts in gut microbiota structure that coincide with their annual dietary changes (Maurice et al., 2015). Also, in wild Mexican black howler monkeys, temporal changes in the relative abundance of gut bacteria strongly correlated with dietary variations (Amato et al., 2015). Another study on *Eulemurs* showed that differences in diet in geographically separated population strongly influence intestinal microbiota (de Winter et al., 2018b). Hence, seasonal diet shifts are likely to explain most of the variation in microbiota in lemurs across seasons.

In addition, the microbial alpha richness from lemurs in Ankarafantsika NP (Crowley et al., 2012) was higher in the early dry season compared to the early wet season. Over the dry season, lemurs experience conditions of relatively low temperatures and food and water restriction, especially in the dry western parts of Madagascar. This nutritional stress may result in a narrower diet and the microbiota would be more specifically adapted to the food items available. This narrower diet during the dry season could therefore explain the gradual decrease in microbiota richness that we observed. Such dietary change might lead to an altered microbial composition, which potentially facilitates the digestion of specific food items. It is tempting to speculate that this could also lead to an increased caloric intake, which might contribute to an increased fitness of both the host and microbiota (Maurice et al., 2015).

The presence of different fruit trees results in large dietary differences across populations (Sato et al., 2014; Styger et al., 1999). For example, the four most predominant food items consumed by *E. fulvus* in Ankarafantsika in the early wet season, were *Buddleja madagascariensis*, *Psychotria*sp., *Vitex perrieri*, and *Diospyros tropophylla*) (Sato, 2013; Sato et al., 2014), species that do not occur in Nosy Tanikely nor Andasibe NP (Styger et al., 1999). Furthermore, introduced mango trees

(*Mangifera indica*) were only consumed at Nosy Tanikely during our study. However, there is also some dietary overlap across populations, i.e., *Dichapetalum leucosia* and *Landolphia myrtifolia* were consumed by *E. fulvus* in both Ankarafantsika and Andasibe NP. Despite the overlap in some fruit species, the geographically separated populations of this lemur species showed strong dietary differences, probably leading to major variations in microbiota composition in these populations.

We found a slight, but not significant, indication that parasite infections in the dry regions of Madagascar showed larger seasonal contrasts compared to the eastern rainforest. Another study also found a higher parasite richness in areas with a large precipitation range throughout the year (Guernier et al., 2004). Many parasites require a certain temperature and humidity to complete their life cycles (Guernier et al., 2004) or as microhabitats for their larva (Froeschke et al., 2010). The drier conditions towards the end of the dry season can prevent egg development and can lead to desiccation of the fragile eggs (Nunn and Altizer, 2006). However, some related nematode species are able to survive such short periods of drought by entering a state of hypobiosis, until humidity conditions improve to the point where free-living larval stages can survive (Brooker et al., 2006). In addition to these direct seasonal influences on parasites, the lemur host influences these infection patterns as well. The host's behaviour, resource use, and diet in general are considered as major determinants of host exposure to parasites (Nunn and Altizer, 2006). It was also experimentally established that host foraging ecology has important consequences for the exposure to and transmission of parasites (Luong et al., 2014). Food scarcity for lemurs is relatively high towards the end of the dry season (Tecot, 2008; Wright et al., 2005) and the associated nutritional stress can have a repressive effect on the host's immune system, which may result in a higher susceptibility to parasite infection (Chapman et al., 2005).

Seasonal changes in lemur reproductive status can also lead to changes in parasite infections patterns (Clough, 2010). The early dry season coincides with the mating season of *Eulemurs* (Overdorff and Johnson, 2003), and more frequent physical contact both within and between lemur groups during this period may enhance parasite infection (Clough, 2010). Besides, androgen and glucocorticoid levels of males and oestrogen levels of females increase during the mating season, which can lead to a higher susceptibility to parasite infections due to their repressive effect of such hormones on the immune system (Ostner et al., 2008). Furthermore, the early wet season coincides with the weaning season, a season that is energy demanding, especially for lactating females. These behavioural and physiological differences may lead to differences in parasites infection status across different seasons. It is likely that, because of all these factors that influence parasite infections, we did not find a stronger effect of seasonality in areas with stronger seasonal contrasts.

We also did not find an interactive effect of the two nematode species as

co-infection appears to be independent. *Lemuricola* and *Callistoura* spp. colonise distinct parts of the gastrointestinal tract of their hosts, the caecum-colon and ileum-colon respectively (Irwin and Raharison, 2009), which can explain the lack of interactions between these two species.

DISTURBANCE

We hypothesised that the microbiota composition would be altered and parasite infection prevalence would be increased in lemurs whose habitat is restricted to more intensely logged forests. For the microbial composition, we found statistically significant variation between samples taken at a previously logged and at a less disturbed site. Moreover, a higher richness of microbial consortia was observed in the logged area. Although only few studies have addressed the impact of anthropogenic disturbance on gut microbiota of wild primates, most studies seem to contradict our findings. For example, habitat disturbance was reported to lead to reductions in the gut microbial diversity of howler monkeys (Amato et al., 2013) and a similar pattern was found in Udzungwa red colobus monkeys (Barelli et al., 2015). These results may reflect a general pattern of habitat degradation and reduced diversity in the ecological pool of microbial taxa available to colonise hosts (Amato et al., 2013). However, the number of studies in this field are very limited. In addition, the type and intensity of anthropogenic disturbance and the forests' regeneration time may be important as well (Johns and Skorupa, 1987). Logging in our sites occurred nearly thirty years ago and sites have been regenerating since (Wright and Andriamihaja, 2002), which can explain the deviating patterns that were found in this study. Nevertheless, these forests still differ to a large extent in their structural characteristics, as well as tree species composition (de Winter et al., 2018a), which may explain the differences in microbiota composition we found.

Remarkably, we found a relatively high abundance of Cyanobacteria in the *Eulemur* population in the less disturbed compared to the previously logged site. Sequences identified as Cyanobacteria are most probably derived from their non-photosynthetic gut dwelling siblings (Di Rienzi et al., 2013). Even though they are part of the normal gut microbiota of mammals, it is not clear what role they play in intestinal ecosystems.

Concerning parasites, the prevalence of *Lemuricola* spp. was significantly higher in the more intensely disturbed site compared to the less disturbed site, while *Callistoura* spp. prevalence showed no such pattern. Selective logging results in a suite of alterations that may increase infection risk and susceptibility to certain parasite infections in resident populations (Gillespie et al., 2005). For example, studies on howler monkeys have reported higher GI parasite diversity and abundance in primates inhabiting degraded areas to those in less disturbed areas (Vitazkova and

Wade, 2007). The depletion of the GI microbiota in degraded environments may explain these patterns. However, other studies show only minimal effects of disturbance on patterns of intestinal parasite infection (Martinez-Mota, 2015). As mentioned above, our logged forest site has been regenerating over decades, and it seems that lemurs have been able to adapt to differences in food availability and forest structural differences accordingly (de Winter, et al. 2018a). As eggs of *Lemuricola* spp. are deposited in the perianal region of their host (Irwin and Raharison, 2009), body contact and grooming behaviour may be important factors in explaining the prevalence of this nematode species within a population. Interaction rates and local lemur densities may be increased and home ranges may be restricted in the more intensely logged forest, which has been shown to increase parasite infection risks (Arneberg, 2002; Chapman et al., 2005). This may explain the higher *Lemuricola* spp. prevalence we found in these forests.

MICROBIOTA AND PARASITES

Our results revealed correlations between parasites and bacterial microbiota composition, as lemurs that showed infections with *Callistoura* spp. and/or *Lemuricola* spp. exhibited distinct profiles of GI microbiota composition compared to non-infected individuals. This indicates that variation in gut microbiota composition is related to nematode presence. This finding is in line with several other studies that observed a relationship between microbiota and GI parasites (Houlden et al., 2015; Kay et al., 2015; Lee et al., 2014; Maurice et al., 2015; Morton et al., 2015; Mutapi, 2015). The lemur population in Ankarafantsika NP showed a significantly lower infection prevalence of *Callistoura* spp. compared to lemur populations in other areas and at the same time, this population showed the highest microbiota richness. GI parasites can damage the host's intestinal epithelium or extract nutrients in the GI tract, which can lower the number of different niches for specific microbial taxa or functional groups (Li et al., 2012). In addition, parasites are able to modify the microbiota through secretory antimicrobial products or by inducing an inflammatory response with potential consequences for the microbial composition (Reynolds et al., 2014). Therefore, parasite-infected lemurs may show a different GI microbiota composition when compared to non-infected lemurs.

In turn, several other studies found that the presence of some nematode species was linked to high microbiota diversity, with potential beneficial consequences for host health (Hayes et al., 2010; Kreisinger et al., 2015; Rausch et al., 2013; Reynolds et al., 2014; Walk et al., 2010). It is assumed that the immune system is regulated by the GI microbiota, but also that GI nematodes can alter the bacterial composition and structure, thereby creating conditions that can facilitate nematode infestations (Hayes et al., 2010). Although it has been shown that some parasites change environmental

conditions prevailing in the intestine, and thus also influence microbial habitats, the exact relations between parasites and the microbiota remain unclear (Reynolds et al., 2015). Most parasite species, and directly transmitted parasites in particular, co-evolve in association with only a few host species and adapt to the host gut environment and diet, resulting in host-driven diversification (Pedersen et al., 2005). We identified the nematodes to the genera instead of the species level. To determine whether the nematodes from the genera *Callistoura* and *Lemuricola* are different species, genetic identification is needed in further studies. Understanding underlying mechanisms is critical for improving our knowledge on parasite-microbe interactions in wild primate populations. Our results suggest that GI parasites potentially have a role in shaping the microbial composition within wild lemur populations or vice versa.

CONCLUSIONS

In conclusion, this study investigated the impact of seasonality and past logging on host-associated parasite infections, faecal bacterial communities, and correlative patterns between these GI inhabitants in geographically separated *Eulemur* populations. Our results show that seasonal differences and past logging events significantly contributed to explaining the observed temporal variations in parasite infections and microbial diversity. The variation in microbiota composition at the genus level showed a significant correlation with the presence of parasites, suggesting a relationship between GI parasites and microbiota composition under natural conditions. The factors that influence microbiota composition and presence of parasites may in turn affect host nutrition, behaviour, and health. These findings likely apply to other wild mammal communities as well, and we believe it is important to consider the potential role of microbiome-parasite associations on the hosts' GI stability, health, and survival.

CHAPTER 6

DETERMINING *MHC-DRB* PROFILES IN WILD POPULATIONS OF THREE CONGENERIC TRUE LEMUR SPECIES BY NON-INVASIVE METHODS

de Winter, I.I., Qurkhuli T., de Groot, N., de Vos-Rouwelaar, A.J.M., van Hooft W.F., Heitkönig, I.M.A., Prins, H.H.T., Bontrop, R.E., Doxiadis, G.G.M.

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ABSTRACT

The major histocompatibility complex (MHC) is a highly polymorphic and polygenic genomic region that plays a crucial role in immune-related diseases. Given the need for comparative studies on the variability of immunologically important genes among wild populations and species, we investigated the allelic variation of MHC class II *DRB* among three congeneric true lemur species that diverged up to 4.5 million years ago: namely, the red-fronted lemur (*Eulemur rufifrons*), the red-bellied lemur (*E. rubriventer*), and the black lemur *(E. macaco*). In a non-invasive manner, we collected hair and faecal samples from these species across different regions in Madagascar. We assessed *DRB* exon 2 polymorphism with a newly developed primer set, amplifying nearly all non-synonymous codons of the antigen binding sites. Twenty-six *DRB* alleles from 45 individuals (17 alleles from *E. rufifrons* (*N* = 18); 5 from *E. rubriventer* (*N* = 7); and 4 from *E. macaco* (*N* = 20)) have been defined. All detected alleles are novel and show a high level of nucleotide (26.8%) and non-synonymous codon polymorphism (40.9%). In these lemur species, we found neither a duplication of *DRB* genes nor sharing of alleles among sympatric groups or allopatric populations of the same species. The non-sharing of alleles may be the result of a geographic separation over a long time span and/or different pathogen selection pressures. We found dN/dS rates > 1 in the functionally important antigen binding sites, providing evidence for balancing selection. Especially for small and isolated populations, quantifying and monitoring *DRB* variation is recommended to establish successful conservation plans that mitigate the possible loss of immunogenetic diversity in lemurs.

INTRODUCTION

The major histocompatibility complex (MHC) is a highly polymorphic and polygenic genomic region, and diversity at this complex is considered an important measure of immunocompetence (Kelley et al., 2005; Klein, 1986; Piertney and Oliver, 2006). The MHC class I and II genes play a crucial role in innate and adaptive immunity, having a major impact on disease resistance (Oliver and Piertney, 2006; Rioux et al., 2009; Savage and Zamudio, 2011; Schwensow et al., 2007). These genes encode cell-surface receptors that present antigens derived from intra- and extracellular parasites and pathogens to T lymphocytes that may consequently initiate an immune response (Germain, 1994; Rammensee et al., 1995). Therefore, the MHC genotype determines the diversity of parasites and pathogens that can be recognised, and correlations between particular MHC alleles, high allelic diversity, and number of MHC genes on the one hand and disease resistance on the other have been demonstrated across vertebrate taxa (Briles et al., 1983; Langefors et al., 2001; Schad et al., 2005). For this reason, genetic variation in functionally important MHC gene families plays a central role in vertebrate immunity and in the viability and long-term survival of wildlife populations (Piertney and Oliver, 2006; Radwan et al., 2010; Siddle et al., 2007).

The MHC class II region varies between species in the number and presence of genes (Kelley et al., 2005). Some MHC gene families are highly variable, not only in number of alleles but also in the extent of sequence variation between alleles. Variation in the MHC is generated at multiple levels. Animals interact with their immediate environment and are exposed continuously to parasites and pathogens. Selection processes increase resistance to such pressures by generating allelic variation through mutation and recombination, which is well reflected in the diversity patterns of MHC genes (Kurtz et al., 2006; Penn et al., 2002). MHC polymorphism can therefore be similar among species due to their co-ancestry (Figueroa et al., 1988; Klein, 1987; McConnell et al., 1988).

The high allelic variation is maintained by balancing selection (Grogan et al., 2016; Piertney and Oliver, 2006; Sommer, 2005; Spurgin and Richardson, 2010), with an increased ratio of non-synonymous over synonymous substitutions at the functionally important antigen binding sites (ABS) (Fijarczyk and Babik, 2015; Garrigan and Hedrick, 2003). Balancing selection can be attributed to two processes. First, it can occur when heterozygous individuals are favoured, likely as a result of their ability to respond to a broader array of pathogens than homozygotes (Doherty and Zinkernagel, 1975). Second, frequency-dependent selection assumes a co-evolutionary arms race between hosts and pathogens (Takahata and Nei, 1990). Polymorphism is maintained when rare alleles are more resistant to pathogens, and are consequently favoured and spread through the population. As soon as parasites have developed antigenicity for these antigens, new, rare alleles will have selective advantage and lead to genetic diversity

in populations (Borghans et al., 2004; Takahata and Nei, 1990).

Gene duplications and deletions can occur in MHC regions in many primate species (Klein et al., 1993; Kulski et al., 1999; Nei et al., 1997), which result in copy number variation (CNV) among individuals (Doxiadis et al., 2010; Kulski et al., 1999; Slierendregt et al., 1994) and between species (Adams and Parham, 2001; Kelley et al., 2005). However, selection against deleterious gene duplications in the MHC operates as well (Shiina et al., 2006), and CNV can also be lost due to genetic drift (Eimes et al., 2011; Schrider and Hahn, 2010).

For decades, much has been known about MHC variation, structure, and evolution in humans, captive non-human primates, and other model organisms (Klein, 1986; Root-Bernstein, 2005). Most of these previous studies focused on the second exon of the MHC II *DRB* gene(s), because this exon encodes functionally important peptides of the antigen binding site (ABS) (Harf and Sommer, 2005). Since exon 2 has been described to be the most polymorphic part in many class II genes, it is therefore assumed to be involved in the susceptibility to specific pathogens (Brown et al., 1993). The class II genes are physically linked, and alleles on these genes are in strong linkage disequilibrium (Marsh et al., 1999). Therefore, *DRB* gene diversity patterns can be a good indicator for the genetic variation in other class II genes, and even for other less closely linked MHC genes (Kelley et al., 2005). In addition, the MHC system is one of the few genetic systems where balancing selection has been revealed in humans and rodents under laboratory conditions, and where studies on various captive or semi-captive breeding primate populations exist (Lafont et al., 2007; Schwensow et al., 2007), although comparatively little research has been done on wild populations of mammals, and on primates in particular (e.g., Tung et al. 2015). Captive populations usually guarantee an easy access to blood or other tissue types, which results in the high quality and quantity of DNA extracts. In contrast, studies on the variation in the MHC system of free-ranging wild animal populations, including lemurs, are still rare compared to those on captive animals (Bernatchez and Landry, 2003; Kaesler et al., 2017). For animal welfare or technical reasons, such studies often rely on non-invasive sampling for genetic and molecular ecology research. This involves challenges to error-free genotyping from a low quantity of low-quality materials.

Over the past century, lemurs have experienced major declines in range size, and nearly half of all lemur species in Madagascar are threatened with extinction as a result of anthropogenic habitat disturbance and unsustainable hunting (Mittermeier et al., 2010). This study focuses on three species of true lemurs, genus *Eulemur*, family Lemuridae: the red-fronted lemur (*Eulemur rufifrons*), the red-bellied lemur (*Eulemur rubriventer*), and the black lemur (*Eulemur macaco*), each of which diverged about 4.5 million years ago (mya) (Markolf et al., 2013; Yoder, 2007). Two of these species (*E. rufifrons* and *E. rubriventer*) live sympatrically but do not hybridise, and all other

Eulemur populations are geographically isolated (Markolf et al., 2013). Owing to their highly ecological flexibility, different species of the genus *Eulemur* occupy most biogeographic regions of Madagascar (Johnson, 2006), including some of the smaller peripheral islands (Colquhoun, 1993). At the same time, they show many similarities in morphology and physiology, as they are genetically closely related (Markolf et al., 2013). Therefore, this genus provides an opportunity to assess the importance of environmental differences in MHC variation as well as the role of balancing selection, which needs to be clarified in *Eulemurs*.

The specific objective of this study was to investigate the allelic variation of the *DRB* gene of the three species of wild true lemurs in different congeneric species across the island Madagascar. In this study, we used a new set of primers that amplifies elongated fragments including amino acids 9 – 13 of exon 2, which represent one of the most important antigen binding motifs of the beta chain of *DRB*. As a result, this study provides a baseline from which to expand further exploration of lemur MHC in conjunction with wildlife diseases, demographic processes, and other selective forces.

METHODS

STUDY SPECIES

True lemurs (genus *Eulemur*, family Lemuridae) are morphologically much alike, and are medium-sized (body and tail length 30 - 50 cm, 2 - 4 kg) arboreal primates that occasionally move quadrupedally on the ground. Their diet consists primarily of fruits, flowers, and leaves (Markolf, 2013), although they are all capable of adding alternative food sources such as invertebrates to their diet. This study focuses on three *Eulemur* species: the red-fronted lemur (*Eulemur rufifrons*), the red-bellied lemur (*E. rubriventer*), and the black lemur *(E. macaco*). The main difference between these *Eulemur* species is their social organisation, including group size: *Eulemur rufifrons* and *E. macaco* live in multi-male, multi-female groups of four to 18 individuals (Erhart and Overdorff, 2008; Overdorff, 1996), whereas *E. rubriventer* lives in small monogamous groups from two to five individuals (Overdorff, 1996). All three species are listed in the IUCN Red List of Threatened Species: *Eulemur rufifrons* as 'near threatened', and *E. rubriventer* and *E. macaco* as 'vulnerable' (Andriaholinirina et al., 2014).

STUDY SITE

We collected biological materials from three different lemur species in four different field sites across Madagascar. We collected samples from *Eulemur rufifrons* and *E. rubriventer* in Ranomafana National Park (NP) (N: -21.32, E: 47.40), samples from *E. rufifrons* in Isalo NP (N -22.47, E: 45.26) and Kirindy Forest (N: -20.07, E: 44.66), and samples from *E. macaco* on Nosy Komba (N: -13.46, E: 48.35) (Fig. 6.1). Kirindy Forest

and Isalo are located on the western side of Madagascar, and consist of dry deciduous forest with pronounced seasonality. These western regions have a higher annual mean temperature than the eastern rainforests, and receive less rainfall (Goodman et al., 2005). Ranomafana NP is a humid rainforest located on the eastern side of Madagascar. The island Nosy Komba is located in the north-west of Madagascar, and is covered with tropical vegetation.

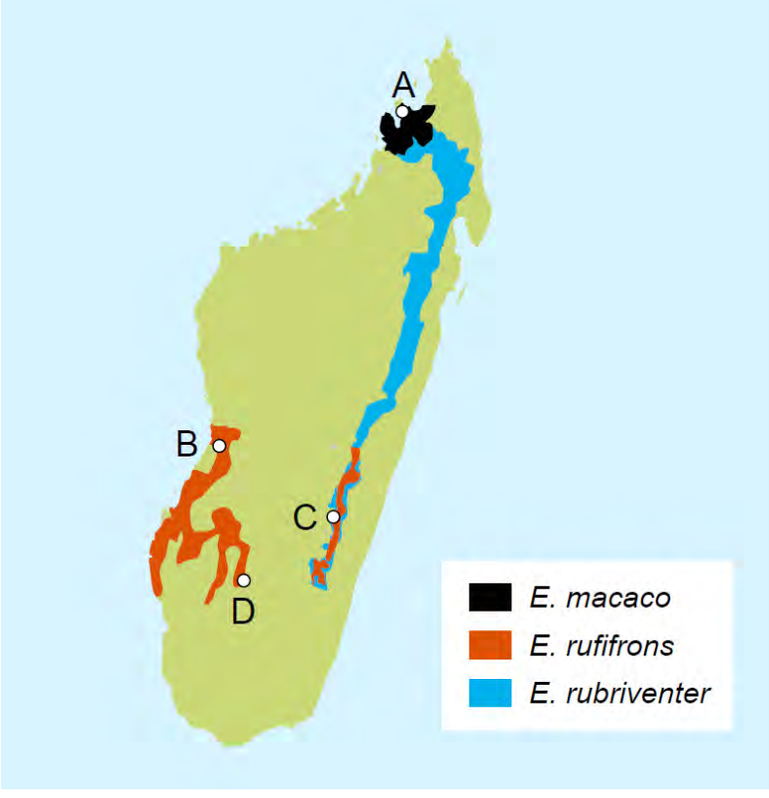

Figure 6.1: Study sites and geographic ranges of the study species. Map of Madagascar with the geographic ranges of the three study species: *Eulemur macaco*, *E. rufifrons*, and *E. rubriventer* and the corresponding sites where samples were collected: A) Nosy Komba; B) Kirindy Forest; C) Ranomafana NP; and D) Isalo NP.

SAMPLE COLLECTION

In a non-invasive manner, we collected samples (N = 45 individuals, N = 51 faecal, and N = 20 hair samples) from individuals between October 2013 and May 2014. Immediately after animals had defecated, fresh faecal samples (3 to 4 g) of adult lemurs were collected with a pincer that we cleaned with ethanol to avoid contamination. We aimed to sample all adult individuals within a social group, and prevented duplication by identifying each individual on the basis of its morphology. From some individuals, both hair and faecal samples were collected. Within 12 h after collection, we stored the collected samples in 15 mL tubes containing 15 g of silica beads to desiccate the faeces (Wasser et al., 1997). We stored these samples in the

shade at ambient temperatures until further analyses by colleagues in the Department of Comparative Genetics & Refinement, Biomedical Primate Research Centre (BPRC) in the Netherlands. In addition to faecal samples, hair was collected opportunistically when possible, and stored in small zip-lock bags. When the lemurs approached closely enough, a tuft of hair was removed from the hip region. Sample collection and export was approved by the trilateral commission (CAFF/CORE) in Madagascar (permits 297/13 and 143/14/MEF/SG/DGF/DCB.SAP/SCBSE).

DNA EXTRACTION

Total DNA was extracted from faeces by using the QIAamp DNA Stool kit (Qiagen) according to the manufacturer's guidelines, with a few modifications (Nsubuga et al. 2004), which include the following. At the start of the extraction, 1) we covered the silica beads in 15-ml tubes containing the dried faecal samples with 1.5 to 2 ml of ASL buffer; 2) the tubes were shaken for 12 to 16 h at 25°C; 3) the supernatant was fully removed from the preservation tubes to extract the DNA; 4) at the end of the extraction, the recommended step of 1 min centrifugation at full speed in a new collection tube was applied; 5) 50 l of AE buffer was used for elution; and 6) incubation at room temperature for 20 min was followed by centrifugation at full speed for 2 min. DNA from hair was extracted by using the Gentra Puregene tissue kit (Qiagen) according to the manufacturer's guidelines. DNA quality and quantity were estimated by absorbance at 260/280 nm on the ND-1000 NanoDrop®.

PCR REACTION FOR *DRB* EXON 2

A 213-bp fragment of *DRB* exon 2 was amplified by PCR using a generic 5 *DRB*-exon 2 primer CGT GTC CCC ACA GCA CGT TTC (Doxiadis et al. 2006) together with the 3'JS2 primer GAT CCC GTA GTT GTG TCT GCA (Schad et al. 2004). The PCR reactions were performed in a 50 μl volume containing 5 units of Platinum *Taq* polymerase (Invitrogen, Paisley, Scotland) with 0.2 μM of each primer, 5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 x PCR buffer (Invitrogen, Paisley, Scotland), and 50-200 ng DNA. The cycling parameters were a 2- min at 94°C initial denaturation step, followed by 3 cycles of 90 s at 94°C, 90 s at 60°C, and 90 s at 74°C. This programme was followed by 32 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 74°C. A final extension step was performed at 72°C for 7 min.

CLONING AND SEQUENCING

PCR products were purified using a geneJet Gel Extraction Kit (Thermo Scientific™) and the purified amplicons were cloned into the pJET vector using the CloneJET PCR cloning kit, both according to the manufacturer's guidelines (Thermo Scientific™). Next, the cloned amplicons were transformed in *Escherichia coli* XL1-blue cells by using the TransformAid Bacterial Transformation Kit (Thermo Scientific™). Per animal,

24 to 48 bacterial clones were picked, and plasmid DNA was isolated using a standard mini-preparation procedure. The purified plasmid DNA was sequenced on the ABI 3500 genetic analyser (Applied Biosystems, Foster City, USA). The sequencing reaction was performed by using 2 M pJET primer, 1 l BigDye terminator, and 2 l of 5x sequencing buffer in a total volume of 10 l (Thermo Scientific™). The resulting sequences were analysed using the Sequence Navigator programme (Applied Biosystems, Foster City, USA). MHC sequences were revised manually by applying the Lasergene 12 SeqMan Pro Sequence Alignment Editor.

ALLELE DISCOVERY AND NOMENCLATURE

All sequences were compared in BLAST at GenBank (National Centre for Biotechnology Information, NCBI), and turned out to be novel and related to *Eulemur DRB* exon 2. Only sequences with an identity higher than 95% to already published lemur *DRB* alleles were considered to be of lemur origin, and were selected for further analysis. Furthermore, only sequences that were detected at least two times, either in two different PCRs of the same sample or in two different animals, were accepted as being new alleles. The alleles were named numerically based on general principles used for the IPD-MHC 2.0 database (Maccari et al., 2017). We deposited all alleles in GenBank, and they were given the accession numbers MF682987- MF683012.

PHYLOGENETIC ANALYSIS

We constructed a Neighbor-Joining phylogenetic tree to show phylogenetic relationships among *DRB* alleles of *E. rufifrons*, *E. rubriventer*, and *E. macaco*, with evolutionary distances computed according to the Kimura 2-parameter method (Saitou and Nei 1987). We used a bootstrap consensus tree inferred from 2000 replicates, and included both transitions and transversions, assuming rates among sites to have a gamma distribution, with a Gamma parameter set to 1. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 26 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 213 positions in the final dataset. Evolutionary analyses were conducted in MEGA V.5 (Tamura et al. 2011).

DN/DS CALCULATIONS

We calculated the relative rate of non-synonymous (dN) and synonymous (dS) substitutions (Nei and Gojobori, 1986) with the Jukes-Cantor correction (Jukes et al., 1969) for multiple hits in MEGA. Substitution rates were calculated for the overall

sequences, and then separately for ABS and non-ABS. Concordance with ABS in human MHC molecules was assumed, with the following beta chain residues: 9, 11, 13, 28, 30, 32, 37, 38, 47, 56, 60, 61, 65, 68, 70, 71, 74, 75. The codon for residue 78 was missing in the derived *DRB* sequences, as it was a partial exon. In addition to these residues, two other residues (26 and 58) have been reported as being involved in antigen binding in lemurs (Schad et al., 2004). Therefore, they were also included in estimating the ABS substitution rate. Statistical differences in dN/dS rates were tested with a *Z*-test. For all calculations, the alpha level was set at 0.05.

RESULTS

DRB ALLELE DEFINITION AND VARIATION

A total of 26 *DRB* alleles could be identified among 45 individuals of *E. rufifrons* (*N* = 18), *E. rubriventer* (*N* = 7), and *E. macaco* (*N* = 20, Tables 6.1, 6.2) . *Eulemur rufifrons* showed the highest allelic variation, with 17 different *DRB* alleles (*Eufr-DRB∗01-17*) defined in 18 animals. Most individuals of this species lived in Kirindy Forest (Table 6.2, Fig. 6.1), and, accordingly, most alleles are defined in these animals (Table 6.1). In three animals from Isalo NP, three other *DRB* alleles were determined along with another two alleles in the two individuals from Ranomafana NP. *Eulemur rubriventer* showed the second highest allelic variation, with five alleles (*Euru-DRB∗01-05*) among eight animals, whereas *E. macaco* was least polymorphic for its *DRB* gene, with only four alleles (*Euma-DRB∗01-17*) defined in the 20 individuals genotyped (Tables 6.1, 6.2).

Eufr-DRB was characterised by high polymorphism, with most alleles being observed in just one or two animals, whereas *Euru-DRB* but especially *Euma-DRB* alleles were detected in far more animals. Five to ten *E. macaco* individuals shared the same allele, with the exception of *Euma-DRB∗04*, which was observed in one animal only (Table 6.1). Most individuals were heterozygous (Table 6.2; observed heterozygosity: *Eulemur rufifrons*: 0.78, *E. rubriventer*: 0.57, and *E. macaco*: 0.55). None of the animals showed more than two *DRB* alleles, indicating that the *DRB* gene is not duplicated in these species.

To visualise the phylogenetic affinities among species, we built a neighbour-joining tree, including the 26 different *DRB* alleles (Fig. 6.2). The branches in the resulting phylogenetic tree may indicate different *DRB* lineages (Fig. 6.2A-G). Each of the three species possesses one allele that clusters separately from the others, indicating evolutionarily long divergence times. The allele *Euru-DRB04-RNP*, found in *E. rufifrons* in Ranomafana NP, forms a single branch (Fig. 6.2E), whereas *Eufr-DRB01-KIR* and *Euma-DRB02-NK*, found in *E. rufifrons* in Kirindy Forest and *E. macaco* on Nosy Komba, respectively, form separate branches within one cluster (Fig. 6.2G). The other clusters were present in all three lemur species. Only a few *DRB*

alleles within a species appear to be closely related. For example, the alleles *Eufr-DRB02* and *Eufr-DRB05* showed only two nucleotide differences, and were isolated from animals from the same location, Kirindy Forest (Fig. 6.2B). In contrast, we identified closely related alleles with just two nucleotide differences from two different populations of the species *Eulemur rufifrons*: *Eufr-DRB12-KIR* from Kirindy Forest and *Eufr-DRB15-IS* from Isalo NP (Fig. 6.2C). The four alleles of *E. macaco* are located very distantly from each other in three different branches of the phylogenetic tree.

Table 6.1: All detected alleles (*N* = 26) and the specific individual samples.

(a) *Eulemur rufifrons*, Kirindy

#	Allele	Animal ID
1	*Eufr-DRB**01	K1RF, K5RF
2	*Eufr-DRB**02	K7RF, K2RF
3	*Eufr-DRB**03	K10RF
4	*Eufr-DRB**04	K1RF, K25RF, K4RF, K9RF
5	*Eufr-DRB**05	K25RF
6	*Eufr-DRB**06	K6RF
7	*Eufr-DRB**07	K27RF, K8RF, K5RF
8	*Eufr-DRB**08	K10RF
9	*Eufr-DRB**09	K7RF, K2RF
10	*Eufr-DRB**10	K11RF, K4RF, K3RF
11	*Eufr-DRB**11	K8RF
12	*Eufr-DRB**12	K3RF, K11RF

(b) *Eulemur rufifrons*, Isalo NP

#	Allele	Animal ID
13	*Eufr-DRB**13	I7RF, I4RF
14	*Eufr-DRB**14	I2RF
15	*Eufr-DRB**15	I4RF, I7RF, I2RF

(c) *Eulemur rufifrons*, Ranomafana NP

#	Allele	Animal ID
16	*Eufr-DRB**16	R90RF
17	*Eufr-DRB**17	R105RF, R90RF

(d) *Eulemur rubriventer*, Ranomafana NP

#	Allele	Animal ID
1	*Euru-DRB**01	R43RB, R29RB, R27RB
2	*Euru-DRB**02	R30RB, R27RB
3	*Euru-DRB**03	R33RB, R29RB, R35RB
4	*Euru-DRB**04	R30RB
5	*Euru-DRB**05	R33RB, R34RB, R55RB, R35RB

(e) *Eulemur macaco*, Nosy Komba

#	Allele	Animal ID
1	*Euma-DRB**01	NK7, NK13, NK19, NK9, NK14, NK2
2	*Euma-DRB**02	NK21, NK16, NK7, NK17, NK9 NK3, NK1EMA, NK4, NK12, NK1
3	*Euma-DRB**03	NB1EMA, NK3, NK4, NK16, NK22, NK2, NK12
4	*Euma-DRB**04	NK19

Table 6.2: Individual MHC class II *DRB* exon 2 genotypes for 45 different lemurs.

(a) *Eulemur rufifrons*, samples from Kirindy Forest, Isalo NP, and Ranomafana NP

	Kirindy Forest	**Allele 1**	**Allele 2**
1	K1RF	*Eufr-DRB* 01	*Eufr-DRB* 04
2	K2RF	*Eufr-DRB* 02	*Eufr-DRB* 09
3	K3RF	*Eufr-DRB* 10	*Eufr-DRB* 12
4	K4RF	*Eufr-DRB* 04	*Eufr-DRB* 10
5	K5RF	*Eufr-DRB* 01	*Eufr-DRB* 07
6	K6RF	*Eufr-DRB* 06	
7	K7RF	*Eufr-DRB* 02	*Eufr-DRB* 09
8	K8RF	*Eufr-DRB* 07	*Eufr-DRB* 11
9	K9RF	*Eufr-DRB* 04	
10	K10RF	*Eufr-DRB* 03	*Eufr-DRB* 08
11	K11RF	*Eufr-DRB* 10	*Eufr-DRB* 12
12	K25RF	*Eufr-DRB* 04	*Eufr-DRB* 05
13	K27RF	*Eufr-DRB* 07	

	Isalo NP	**Allele 1**	**Allele 2**
14	I2RF	*Eufr-DRB* 14	*Eufr-DRB* 15
15	I4RF	*Eufr-DRB* 13	*Eufr-DRB* 15
16	I7RF	*Eufr-DRB* 13	*Eufr-DRB* 15

	Ranomafana NP	**Allele 1**	**Allele 2**
17	R105RF	*Eufr-DRB* 17	
18	R90RF	*Eufr-DRB* 16	*Eufr-DRB* 17

(b) *Eulemur rubriventer*, samples from Ranomafana NP

	Ranomafana NP	**Allele 1**	**Allele 2**
1	R43RB	*Euru-DRB* 01	
2	R33RB	*Euru-DRB* 03	*Euru-DRB* 05
3	R34RB	*Euru-DRB* 05	
4	R55RB	*Euru-DRB* 05	
5	R29RB	*Euru-DRB* 01	*Euru-DRB* 03
6	R30RB	*Euru-DRB* 02	*Euru-DRB* 04
7	R27RB	*Euru-DRB* 01	*Euru-DRB* 02

(c) *Eulemur macaco*, samples from Nosy Komba

	Nosy Komba	**Allele 1**	**Allele 2**
1	NK21	*Euma-DRB* 02	
2	NK16	*Euma-DRB* 02	*Euma-DRB* 03
3	NK7	*Euma-DRB* 01	*Euma-DRB* 02
4	NK17	*Euma-DRB* 02	
5	NK3	*Euma-DRB* 02	*Euma-DRB* 03
6	NK1EMA	*Euma-DRB* 02	
7	NB1EMA	*Euma-DRB* 03	
8	NK22	*Euma-DRB* 03	
9	NK13	*Euma-DRB* 01	
10	NK4	*Euma-DRB* 02	*Euma-DRB* 03
11	NK12	*Euma-DRB* 02	*Euma-DRB* 03
12	NK1	*Euma-DRB* 02	
13	NK2	*Euma-DRB* 01	*Euma-DRB* 03
14	NK14	*Euma-DRB* 01	
15	NK9	*Euma-DRB* 01	*Euma-DRB* 02
16	NK10	*Euma-DRB* 02	*Euma-DRB* 03
17	NK19	*Euma-DRB* 01	*Euma-DRB* 04
18	NK23	*Euma-DRB* 02	*Euma-DRB* 03
19	NK5	*Euma-DRB* 02	*Euma-DRB* 03
20	NK15	*Euma-DRB* 02	

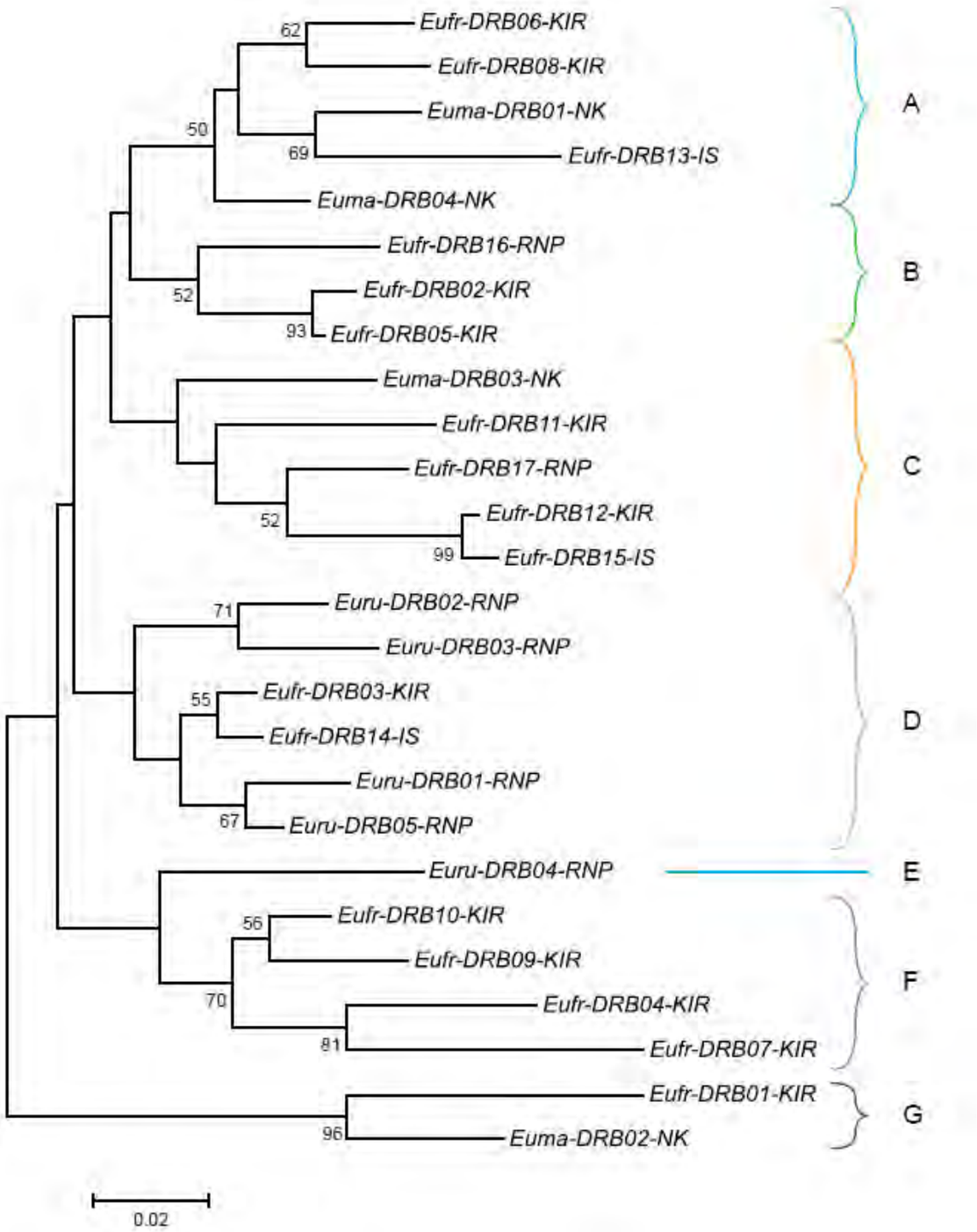

Figure 6.2: Neighbour-joining tree constructed from 26 MHC II *DRB* exon 2 alleles in *Eulemur macaco*, *E. rufifrons*, and *E. rubriventer*. The tree was constructed in accordance with the Kimura-2-parameter model (Kimura, 1980). The percentage of replicate trees in which the associated taxa cluster together in the bootstrap test are depicted in front of a node. Cluster designation is shown next to the branches (letters A-G). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used. An abbreviation of the location where the *DRB* allele has been detected is given in the allele name: Nosy Komba = NK; Kirindy Forest = KIR; Ranomafana NP = RNP, and Isalo NP = IS.

AMINO ACID VARIATION OF THE DR BETA CHAIN

All *DRB* alleles encode for a unique peptide composed of 71 amino acids, indicating a functional *DRB* molecule, with 29 variable amino acids (40.9%). Of the 17 ABS (Table 6.3, indicated by an asterisk), 14 sites (82.4%) are variable, while of the 54 non-ABS only 15 (27.8%) are variable. The ratio of non-synonymous and synonymous substitution rates (dN/dS) at the complete fragments was elevated and was indicative of positive selection ($Z = 3.88$, $P < 0.001$). This can be attributed to the ABS ($Z = 4.58$, $P < 0.001$), as non-ABS did not show a significant positive selection ($Z = 1.27$, $P = 0.103$).

DISCUSSION

DRB ALLELE DEFINITION AND VARIATION

In this study, we obtained elongated *DRB* exon 2 fragments, which allowed us to define the nucleotides encoding nearly all non-synonymous codons of the antigen binding sites. Especially amino acids 9 to 13 are of importance in encoding a peptide motif, which defines the relatedness of *DRB* alleles in humans and non-human primates (e.g., macaques), and are therefore used for lineage definition (Sommer, 2005). These sequences were missing in the *DRB* amplicons of lemur *DRB* sequences published earlier in a mouse lemur species (*Microcebus murinus*) (Schad et al., 2004). Indeed, in the three lemur species analysed in this study, these amino acids were highly polymorphic, and they encode many different motifs that may be useful for phylogenetic purposes when more individuals will have been analysed (Table 6.3). Although the species in our study were all congeneric and some species lived in sympatry, alleles were neither shared between species nor between allopatric populations of a species (i.e., between the three geographically separated *E. rufifrons* populations). *DRB* allele sharing in evolutionarily related species with a divergence time of less than 1.5 mya has been observed: for instance, between rhesus macaques (*Macaca mulatta*) and cynomolgus macaques (*Macaca fascicularis*) (Doxiadis et al., 2006). The absence of allele sharing among the *E. rufifrons* populations may indicate that these populations have been separated for more than 1.5 million years, and different parasite loads may have led to a different *DRB* repertoire. Allele sharing of the three *Eulemur* species with a far higher divergence time of ~4.5 mya was therefore not expected. However, as the three *E. rufifrons* populations are assumed to belong to the same species, it is remarkable that they do not share identical *DRB* alleles. Owing to the low sample size, however, the most plausible explanation would be that not all *DRB* alleles have yet been defined, and therefore low-frequency shared alleles may have been missed. Furthermore, a relatively high number of *DRB* homozygous animals may indicate that due to primer inconsistencies not all alleles have been defined. More animals need to be sampled and analysed, and calculations

of observed versus expected heterozygosity are needed to evaluate whether all *DRB* alleles have been detected.

In a comparison of the different lemur species and populations, *DRB* polymorphism was largest in *E. rufifrons* sampled in a dry deciduous forest (Kirindy Forest). In the lemurs in this forest, the prevalence, species richness, and infection intensities of gastrointestinal parasites are high when compared to other lemur populations in Madagascar (Clough, 2010). Higher and more diverse parasite loads can lead to increased individual MHC diversity (Clough, 2010; Eizaguirre et al., 2011; Harf and Sommer, 2005; Summers et al., 2003). Furthermore, the number of unique alleles present in the population of *E. macaco* on the island of Nosy Komba was more than four times lower than in the *E. rufifrons* population in Kirindy Forest. This might be the effect of a low pathogen pressure on this island, as was also observed on the nearby island Nosy Be (Junge and Louis, 2007). In addition, the low allele diversity within this isolated island population may also be the result of a small founder population, the impact of inbreeding due to the small population size, and the lack of any influx of new individuals (Frankham, 2015).

In contrast to various other non-human primate species, we found no duplication of the *DRB* gene in true lemurs. *DRB* duplication and copy number variation (CNV) of MHC genes is a common phenomenon in vertebrates, and has been reported in many primate species, including chimpanzees (*Pan troglodytes*), orangutans (*Pongo pygmaeus*), macaques (Doxiadis et al., 2006), dusky titis (*Callicebus moloch*) (Trtková et al., 1993), green monkeys (*Chlorocebus sabaeus*) (Rosal-Sánchez et al., 1998), and the Senegal bushbaby (*Galago senegalensis*) (Figueroa et al., 1994). Additionally, other Strepsirrhini primates, including the northern greater galago (*Otolemur garnettii*) and the Senegal bushbaby (*Galago senegalensis*) (Figueroa et al., 1994), have a duplicated *DRB* gene. One lemur species, the grey mouse lemur (*Microcebus marinus*), also shows *DRB* duplication (Go et al., 2002; Huchard et al., 2012). However, gene duplication is rare in lemurs (Averdam et al., 2011; Go et al., 2002), and therefore our results confirm those of previous studies, suggesting that *DRB* duplication is relatively rare in this primate group.

With the exception of two clusters, phylogenetic analysis of the *DRB* alleles of the three *Eulemur* species genotyped in this study does not show clustering of alleles per species (Fig. 6.2 B, F). This may be an indication that most *DRB* alleles are older than the species' divergence time. Instead, we saw an intermingling of alleles from different species. The long branch lengths, at least for some clusters (e.g., Fig. 6.2G), also indicates long evolutionary distances. As a consequence, this finding suggests that these branches represent *DRB* lineages that are shared between the three *Eulemur* species, and are older than the divergence of these species. The only four alleles of *E. macaco* are located at a considerable distance from each other in three branches,

and therefore appear to belong to different lineages. Two animals of *E. rufifrons* and all *E. rubriventer* individuals tested in this study were from Ranomafana NP, which represents a special location. It is situated on the eastern side of Madagascar, and has been isolated from the rest of the island by a major mountainous geographic barrier that runs in a north-south direction over the island. The geographic separation of the populations may explain why *DRB* alleles from animals in Ranomafana NP seem less closely related to other *Eulemur DRB* alleles. The potential different external infection pressures for the separated populations may have played a role in this observation as well. However, to substantiate this suggestion, far more samples have to be analysed in future studies. Additionally, when more samples become available, intron sequences should be analysed that are under less selection pressure than coding sequences in order to gain a better insight into the phylogeny of *DRB* in lemurs (Doxiadis et al., 2012).

AMINO ACID VARIATION OF THE DR BETA CHAIN

The *DRB* genes of all three true lemur species in our study express higher rates of non-synonymous substitutions than expected under neutrality, similar to other findings on lemurs and other primate species (reviewed in Go et al. 2002). We demonstrate a higher dN/dS ratio in the ABS compared to the non-ABS in the respective domains, leading to different amino acid sequences. As expected and confirmed in many other studies (Harf and Sommer, 2005; Schad et al., 2005, 2004), the rate of non-synonymous substitutions did not exceed the rate of synonymous substitutions in the non-ABS. These results indicate balancing selection, leading to high levels of *DRB* diversity and polymorphism. Some studies suggest that this selection pattern is driven by parasites, for example in grey mouse lemurs (Schad et al., 2005, 2004) and mandrills (*Mandrillus sphinx*) (Abbott et al., 2006). As the diversity in MHC II may be linked to the diversity of parasites and pathogens that can be recognised by the host (Briles et al., 1983; Langefors et al., 2001; Schad et al., 2005), the role of parasites in driving *DRB* variation needs to be investigated further.

Table 6.3: Deduced amino acid alignment of the *DRB* exon 2 of *Eulemur macaco*, *E. rufifrons*, and *E. rubriventer*. The sequence alignment starts at amino acid position 8 of exon 2. Dashes indicate identity with the first sequence. Asterisks indicate variable positions. Allele names are given as described for Fig. 6.2

***DRB* allele**								
	* * *		* * * *	* *	*	** *	* * **	
*Eufr-DRB**01-KIR	LEQRKAECHF	YNGTERVRLL	DRYISNGEET	VRFDSDVGEF	RAVTERGVQD	AEYWNSQKDL	LERRRAEVDT	V
*Eufr-DRB**02-KIR	---G------	--------F-	E-HFY-R--F	---------Y	-----L-RGI	--N---L--R	-DYA--A---	Y
*Eufr-DRB**03-KIR	---F-S----	----------	----H-R--F	----------	-----L-RRS	--N------I	-DDA--A---	F
*Eufr-DRB**04-KIR	-Q-F-S----	--------F-	E-H-Y-R--F	M--------Y	-----L-RGI	--NL------	---K--N---	Y
*Eufr-DRB**05-KIR	---G------	--------F-	E-HFY-R--F	---------Y	-----L-RGI	--N---L--I	-DYA--A---	Y
*Eufr-DRB**06-KIR	---A-C----	--------F-	Q--FY-R--Y	----------	-----L-RGI	--NL-----F	-DYL--L---	Y
*Eufr-DRB**07-KIR	-H-F-S----	-------LY-	H--FY-R--Y	----------	-----L-RRS	---F-----F	--QK--N---	Y
*Eufr-DRB**08-KIR	---A-S----	--------F-	Q--FY-R--Y	----------	-----L-RGI	--NL-----R	-DYL-GV---	A
*Eufr-DRB**09-KIR	---A-S----	--------F-	E--FY-R--Y	---------Y	-----L-RRS	--NF--L--R	---K--A---	Y
*Eufr-DRB**10-KIR	---V-S----	--------F-	E--FY-R--Y	---------Y	-----L-RRS	--N------I	---K--S---	Y
*Eufr-DRB**11-KIR	---H-S----	--------F-	E---H-R--L	----------	-----L-RP-	---------R	-DYL-GV---	-
*Eufr-DRB**12-KIR	---G------	----------	L-H-H-R--Y	A--------Y	-----L-RRS	------L--F	-DYL-GA---	-
*Eufr-DRB**13-IS	-H-Y-G----	--------F-	---FY-R--L	M--------Y	-----L-RGI	--NL-----F	-DYL-GV---	-
*Eufr-DRB**14-IS	---F-S----	----------	----H-R--F	---------Y	-----L-RGI	--N------I	-DDA--A---	F
*Eufr-DRB**15-IS	-Q-G------	----------	L-H-H-R--Y	A---------	-----L-RRS	------L--F	-DYL-GA---	-
*Eufr-DRB**16-RNP	---G-S----	--------F-	----H-R--Y	---------Y	-----L-RGI	--NF--L--R	-DYA--A---	F
*Eufr-DRB**17-RNP	---G-S----	----------	Q---Y-R--Y	A---------	-----L-RP-	---------I	-DYL-GV---	-
*Euru-DRB**01-RNP	---V-H----	----------	----H-R--L	---------Y	-----L-RRS	--N------I	-DDA--A---	F
*Euru-DRB**02-RNP	-Q-F-S----	----------	----H-R--F	A--------Y	-----L-RP-	---------I	-DDA--A---	F
*Euru-DRB**03-RNP	-H-F-P----	--------F-	V-H-Y-R--Y	A--------Y	-----L-RP-	---------I	-DDA--A---	F
*Euru-DRB**04-RNP	---V-H----	--------F-	E---Y-R--F	---------Y	-P---L-RP-	---------I	------A---	Y
*Euru-DRB**05-RNP	---V-H----	--------F-	----Y-R--Y	----------	-----L-RRS	--N------I	-DDA--A---	F
*Euma-DRB**01-NK	-Q-F-P----	----------	---FY-R--Y	----------	-----L-RGI	--NL-----T	-DYL-GV---	-
*Euma-DRB**02-NK	---H-P----	--------F-	E---------	----------	----------	---------I	-DDA--S---	F
*Euma-DRB**03-NK	---A-S----	--------F-	----H-R--Y	----------	-----L-RP-	------L-NI	-DDE--A---	-
*Euma-DRB**04-NK	---H-P----	--------F-	---FY-R--Y	----------	-----L-RGI	--NL-----I	-DYL--A---	F

CONCLUSIONS

High polymorphism levels of MHC II genes are considered critical to the long-term survival of animal populations (Edwards and Potts, 1996; Grogan et al., 2017), although species with low diversity could also be viable (Sommer et al., 2002). Like all lemurs, true lemurs face significant anthropogenic threats, including disease pressures, changing climatic conditions, and habitat loss and fragmentation (Reuter et al., 2017; Schwitzer et al., 2013). Many populations have become isolated (Irwin et al., 2010), and we indicate that an isolated population in our study shows a loss of genetic diversity. Studies quantifying *DRB* alleles can assess a species' ability to respond to the many anthropogenic threats they are facing. Especially when comparing different populations and populations with rare or unevenly distributed alleles, a greater sampling effort is needed to detect most of the *DRB* diversity. Sampling within different areas that experience anthropogenic pressures would be very interesting from a conservational perspective. We recommend conservation management to include the analysis of *DRB* polymorphism as a key to the long-term survival of endangered species, such as lemurs in Madagascar. We also recommend investigating the association between *DRB* variation and disease resistance as well as other fitness parameters in threatened populations.

CHAPTER 7

SYNTHESIS: HOW TO KEEP THE RAFT AFLOAT?

In this thesis, I aim to provide an overview of the complex relationships between multiple factors and the presence, behaviour, and health of lemurs in Madagascar. Although lemurs have been extensively studied in the past few decades, an integrated approach to the impacts of multiple natural and anthropogenic habitat alterations on both tropical rainforests and lemur communities was lacking. Especially in this unique island setting, the effects of such disturbances on forests, as well as the presence, behaviour, and health of non-human primates, has remained relatively unexplored and is debated within the scientific community (Gardner et al., 2007). Identifying the natural state of lemur health, the reaction of lemurs to the various challenges they face in more intact as well as disturbed habitats, and identifying ecological problems, is essential to effective primate conservation. The findings of this thesis are therefore of relevance not only for science, but also for the management and conservation of wild lemur populations in Malagasy forests.

Species of the genus *Eulemur* are widely distributed across the many ecosystems of the island of Madagascar; they inhabit a variety of forest environments; and show many similarities in morphology, diet preferences, and social behaviour (Markolf et al., 2013). I non-invasively collected data on four different true lemur species (genus *Eulemur*) in nine geographic locations across Madagascar and at sites within a rainforest with high and low levels of human disturbance. In this thesis, I explore the relationship between natural and anthropogenic impacts on the abundances and health of multiple lemur species across Madagascar, and integrate them with non-invasively collected field data. I quantified forest structure variables and characterised forest composition, performed transect surveys to determine lemur encounter rates and cluster sizes, and collected behaviour data as well as faecal and hair samples. The latter were used to sequence faecal bacterial microbiota, morphologically identify parasite species, and analyse MHC II diversity. In this synthesis, I will also give an overview of the main findings of this thesis, including the answer to all research questions (Box 7.1). In addition, I discuss other challenges that biodiversity conservation in Madagascar is facing in the near future, and provide potential solutions to ensure the long-term survival of lemurs.

THE VALUE OF DISTURBED FORESTS

FOREST DISTURBANCE AND LEMUR PRESENCE

In the first part of this thesis, I assessed the effect of human activities on forest characteristics and lemur presence, to evaluate the regeneration capacity and conservation value of disturbed forests. Logging influences forest structure and animal abundances (Laurance, 2015; Michalski et al., 2007). These changes may favour some species, but they also lead to reduced population sizes and local extinction of others (reviewed in Margules & Pressey 2000). Some studies report that disturbances decrease food availability for forest animals, but other studies found no such effects (reviewed in Gardner et al. 2007). The capacity of forests to regenerate depends on the type of logging the forest has experienced, logging intensity, and on the degree of disturbance to the forest soil and seedbanks.

In contrast to selective logging, completely deforested areas are deprived of both seed dispersers and regenerative power; they can then easily be invaded by alien plants, potentially leading to irreversible effects (Lowry et al., 1997). In contrast, forest recovery is often faster when the disturbance primarily impacts forest canopies, as residual low-level vegetation can serve to promote seedling regeneration and the re-establishment of original forest species (Chazdon, 2003; Guariguata and Ostertag, 2001). The rate and nature of recovery processes and successional trajectories in other tropical forests are thus site specific and depend on the disturbance intensity, land use histories, presence of seed dispersers, environmental conditions, and management practices (Styger et al., 1999).

I described the impact of past selective logging on forest structure and composition, and the encounter rates and cluster sizes of seven sympatric diurnal lemur species living in Ranomafana National Park (NP) in the eastern rainforest of Madagascar. Here, some forest areas had experienced intense logging until the park became strictly protected in 1991 (Wright and Andriamihaja, 2002). Although the forest has been regenerating since the inauguration of the park, my results show that the impact of past logging can still be discerned in several forest structural characteristics and tree species composition. Despite these differences between forest sites, lemur encounter rates and cluster sizes were similar across all sites, even for a large, specialised, frugivorous species. This implies that although the intensely logged forests have not fully recovered to identical pre-logging floristic conditions, they appear to have recovered from a functional perspective into suitable lemur habitat.

LEMUR COEXISTENCE

Anthropogenic disturbances, including selective logging, can create spatial heterogeneity in forests (de Winter et al., 2018a; Questad and Foster, 2008) and can

influence competition and coexistence patterns (López-Gómez and Molina-Meyer, 2006; Roxburgh et al., 2004). In primate species, the geographic coexistence of congeneric species is rare (Houle, 1997), but it is nevertheless relatively common in lemurs (Kamilar et al., 2014). There is still debate in community ecology about the causal mechanism behind the coexistence of closely related lemur species (e.g., Dammhahn and Goodman 2014). Specifically, congeneric species are generally more similar to each other in their biology, ecology, and morphology when compared to more distantly related taxa, while species that share the same habitat should show greater differences in their niches in order to coexist (Sfenthourakis et al., 2005). Therefore, interspecific competition, which becomes more apparent when resources are limited, affects congeneric species more than it affects more distantly related species within a community, thereby constraining the coexistence of such closely related species (Futuyma, 2013). Based on the quantitative behavioural study of habitat selection and direct competition I performed on these two lemur species, I propose that, in addition to niche differentiation (i.e., in resource use, time, and space), large-scale spatial segregation into different areas within a heterogeneous environment, caused by logging, can facilitate the coexistence of closely related species. Thus, in some situations, habitat disturbance can actually enable coexistence patterns, making it a potentially important factor in structuring animal communities. This study adds to the knowledge base for the question why lemurs radiated into so many different species, comprising more than 20 percent of all primate species that are known today. Extreme differences in ecosystems and habitats in Madagascar, in combination with environmental heterogeneity caused by natural and anthropogenic disturbances, have facilitated the coexistence of multiple lemur species, thereby contributing to the extraordinary diversity of this unique primate clade.

LEMUR HEALTH

In the second part of my thesis, I explored different health parameters of lemurs that were exposed to various anthropogenic disturbances. I evaluated the influence of both anthropogenic and natural variation in habitat conditions on the faecal bacterial microbiota composition, parasite infections, and immunocompetence in multiple wild lemur populations. I will discuss the main findings below.

FAECAL MICROBIOTA COMPOSITION

Across Madagascar, lemurs are exposed to natural contrasting environmental circumstances, including biogeographic and seasonal changes in climate conditions as well as to human impacts (Wright et al., 2012). I found that all these elements—biogeographic variation, seasonality, and forest disturbances—impact the faecal bacterial microbiota community composition at operational taxonomic

unit (OTU) level, in lemurs, and are more important than sex, age, or species. Biogeographic conditions and seasonality are associated with dietary composition, which most likely influences the faecal microbiota. Furthermore, I observed a higher diversity in the bacterial microbiota in areas that were more intensely logged compared to less intensely logged areas, which indicates an enlarged diet breadth in such forests. This contradicts studies showing that habitat disturbance leads to reductions in bacterial microbiota diversity in animals (Amato et al. 2013; Barelli et al. 2015) and may indicate that the diversity in food resources is not reduced in these previously logged forests. Alternatively, the higher bacterial microbiota diversity in the logged forests may be the result of the consumption of more alternative food types in the disturbed forests, as has been seen in other primates as well (Riley, 2007). In general, all circumstances that generate changes in food availability in forests can change the faecal bacterial microbiota composition in lemurs. Especially during food scarcity, *Eulemur* spp. have to consume alternative food sources, e.g., leaves, flowers, fungi, and insects (Overdorff, 1993). Incorporating such food sources in their diet leads to changes in the lemur's microbiota composition, which likely facilitates digestion. This makes lemurs more flexible towards a less constant or predictable supply of fruits in the forest.

As humans and their agricultural fields are located in close proximity to the remaining forests in Madagascar, lemurs regularly make use of the widespread introduction of exotic fruit tree species (*personal observation*) and have been seen crop-raiding agricultural fields (e.g., Lafleur & Gould 2009). On one of the research sites, for example, the abundant presence of planted banana and mango trees gave lemurs easy access to these highly sugar-rich food sources. The resulting narrow diet, high in carbohydrates, is a potential driver of the lower alpha diversity we observe in a lemur species living on this island (i.e., *Eulemur fulvus*). The combination of this altered diet and lower daily activity levels, as travel distances in search for food are decreased, may increase the risk of diet-related health problems, including obesity and diabetes (Junge et al., 2009; Kavanagh et al., 2013), and can lead to far-reaching consequences for lemur survival.

GASTROINTESTINAL PARASITES

In addition to effects on microbiota composition, selective logging results in a suite of alterations that may increase infection risk and susceptibility to certain gastrointestinal (GI) parasite infections in resident populations (Gillespie et al., 2005). I examined the presence of GI nematodes using a centrifugation-sedimentation-flotation method and morphologically identified the isolated eggs and worms. In all *Eulemur* populations, I detected species of microphagous pinworms that are part of two helminth genera in the Oxyuridae family: *Callistoura* (Chabaud and Petter, 1959) and *Lemuricola* (Chabaud et

al., 1965). I found these parasites across Madagascar, which confirms that these genera have a broad distribution and no specificity to a particular lemur species within the *Eulemur* genus (Irwin and Raharison, 2009).

The prevalence of *Callistoura* spp. is remarkably high in all populations, but only the prevalence of *Lemuricola* spp. is significantly higher in more intensely logged areas compared to less disturbed parts of the forests. *Callistoura* spp. live and deposit their eggs in the ileum and colon, while *Lemuricola* spp. colonise the colon and caecum and use the perianal region of their host to deposit their eggs (Irwin and Raharison, 2009). Next to the faecal-oral transmission of these parasite species, increased social body contact, including grooming, playing, and mating, can be an important factor in the transmission of *Lemuricola* spp. between lemurs, thereby enhancing the overall prevalence of these parasites. Potential food limitations, restricted home ranges, or close contact with humans and their livestock, can elevate stress levels, potentially making lemurs more susceptible to parasite infections as well (Gillespie and Chapman, 2006). Furthermore, feeding on human plantations or from fruits provided by humans can lead to more encounters with conspecifics that are competing over the present or provided food (Maréchal et al. 2011, *personal observation*). Thus, infection risks may be increased when the transmission of parasites is facilitated by higher inter- and intraspecific interaction rates of hosts, or when elevated stress levels lead to an increased host susceptibility (Arneberg, 2002; Chapman et al., 2005).

Most primate parasites exert long-term, subtle and sub-lethal effects that are often difficult to detect (Goldberg, 2008). Several pinworm infections are known to cause perianal itching, aggressiveness, diarrhea and associated weight loss and dehydration, and even juvenile mortality. Chronic infections with such parasites can be detrimental to host fitness over the long term (Gillespie et al., 2010). Clinical symptoms depend on both the parasite species, the strength of infection, and the condition of the host, and can vary from an asymptomatic to a fatal infection (Kaur and Singh, 2009). Like other studies (Clough, 2010; Irwin and Raharison, 2009), I recorded no apparent clinical symptoms, as none of the lemurs showed obvious signs of disease. It is most likely that species of both *Callistoura* and *Lemuricola* opportunistically inhabit the gut, without harming the host individual, thereby in general being relatively commensalistic (Junge, 2006; Schwitzer et al., 2010). Nevertheless, infection intensity with such parasites have the potential to increase in situations of elevated ecological stress, for example exerted by disturbances to the lemurs' habitat (Gillespie et al., 2010). This may result in stronger health effects and may consequently increase host susceptibility to other diseases, as well (Beldomenico and Begon, 2010).

MICROBIOME-PARASITE ASSOCIATIONS

Bacterial microbiota and parasites co-inhabit the GI-tract and have evolved in close association, suggesting that they are able to influence each other (Kreisinger et al., 2015). Some GI parasites can damage the host's intestinal epithelium or extract nutrients in the GI tract, which can lower the number of different niches for specific microbial taxa or functional groups (Li et al., 2012). In addition, parasites are able to modify the microbiota through secretory antimicrobial products or by inducing an inflammatory response, with potential consequences for the microbial composition (Reynolds et al., 2014). I found a significant correlation in the variation of the faecal microbiota at genus level with the presence of parasites. Based on these results, I suggest an interactive effect between GI parasites and bacterial microbiota composition in wild lemurs.

IMMUNOCOMPETENCE

Major Histocompatibility Complex class II (MHC II) molecules mediate key functions in adaptive immunity by presenting antigen peptides from exogenous proteins, thereby determining the immune responses (Benacerraf, 1988; Ceppellini et al., 1989). MHC II diversity is an important proxy for immunocompetence (Kelley et al., 2005; Piertney and Oliver, 2006). Quantifying MHC II diversity gives an estimation of the animals' ability to respond to threats it faces, including increased pathogen pressure induced by habitat changes. In this thesis, I explored allelic variation in the MHC II *DRB* profile to evaluate the lemurs' potential to recognise a diversity of parasites and pathogens (Langefors et al., 2001; Schad et al., 2005). From the non-invasively collected faecal and hair samples, I successfully genotyped elongated DNA fragments with a newly developed primer set, amplifying nearly all non-synonymous codons of the antigen-binding sites, in three species of brown lemurs in four geographic areas.

All detected MHC II alleles are novel and show a high level of sequence and functional polymorphism. This could be the result of different pathogen-driven adaptive selection pressures on lemur populations living in different geographic areas (Eizaguirre et al., 2011). I also observed that some alleles identified from different lemur species cluster together in the constructed phylogenetic tree, which indicates a similar function for such alleles (Otting et al., 2000). We recorded a higher rate of non-synonymous substitution in the antigen binding sites compared to the non-antigen binding sites of *DRB* alleles. This indicates that balancing selection, driven by pathogens and parasites, generates sequence diversity in the *DRB* gene (Abbott et al., 2006; Schad et al., 2005, 2004). The *DRB* gene shows no signs of duplication, and alleles were not shared among sympatric or allopatric populations of the same lemur species, despite the similar pinworm infections we found in different lemur populations. However, I only isolated and identified macroparasites in the GI

tract, while this is only a minor part of the potential range of infections, such as with other parasites, viruses, and bacteria, where MHC II *DRB* diversity plays a role. In addition, not only the parasite species, but also the intensity of infections could be driving these genetic patterns. For example, an *E. rufifrons* population living in the western dry deciduous forests is known for its extraordinary high prevalence, species richness, and infection intensity of GI parasites when compared to other lemur populations in Madagascar (Clough, 2010). In this particular population, I found the greatest MHCII *DRB* variation among the lemur populations genotyped. This result supports the link between parasite presence and individual MHC II diversity (Eizaguirre et al., 2011; Harf and Sommer, 2005; Summers et al., 2003).

Like all lemurs, *Eulemur* species face significant anthropogenic threats, including habitat loss (Reuter et al., 2017; Schwitzer, 2014). Many populations currently become isolated in forest fragments (Irwin et al., 2010), leading to smaller population sizes and a lower or absent exchange of lemur individuals between such fragments (Hewitson et al., 2011; Laurance, 2008; MacArthur and Wilson, 1967). *Eulemur macaco* is a lemur species that lives in mainland forests of northwest Madagascar and on several islands within the Mozambique channel along Madagascar's coastline (Rabarivola et al., 1991). On one of these islands, Nosy Komba, the number of unique alleles present in this isolated lemur population is much lower than in mainland lemur populations. Several explanations for this low allele diversity exists. First, it might be an effect of a low pathogen and parasite pressure on this island, as was found in another study (Junge and Louis, 2007). Second, as described above, the GI parasite species likely have mild clinical effects in lemurs and may not be of clinical significance (Irwin and Raharison, 2009). Third, being a small founder population, the impact of inbreeding in this small population size and the lack of any influx of lemur immigrants (e.g., Frankham, 2015) could have limited genetic diversity. As forest fragments parallel islands in many ways—for example in terms of forests area and isolation—these processes are likely to play a role in isolated 'islands' of forest on the mainland as well (reviewed in Laurance 2008).

LIMITATIONS AND FUTURE DIRECTIONS

In this thesis, I demonstrated that both natural and ongoing anthropogenic influences impact the habitat of lemurs, as well as their behaviour, interaction patterns, and health. However, many questions concerning these complex interactions and the future existence of lemurs remain unanswered. Here, I will now discuss some limitations of my research, and propose future research directions to extend the scope of my work in future studies.

Overall, all data used in this thesis were collected non-invasively. Data included visual records of animal presence, body condition, and behaviour as well as faecal and hair samples. For many health-related studies, immobilising and capturing animals is

required to obtain blood samples and health measurements. This is quite invasive and can exert a lot of stress on animals. However, the downside of taking non-invasive animal health measurements, is that subclinical signs and sublethal effects could have been missed.

I show that selectively logged forests do not fully resemble pristine forests, even after decades of regeneration, but that these forests nevertheless function as habitat for a diverse lemur community (**chapter 2**). The impact of anthropogenic disturbances on forests depends on many factors, including the intensity of the disturbance, land use histories, the presence of biotic seed dispersers, environmental conditions, and management practices (Styger et al., 1999), as well as the tolerance of the specific species or wildlife community considered. Although for some species resource availability may decrease after disturbances, other species may even benefit from specific disturbance-induced changes. Therefore, more local studies are needed to evaluate the situation in multiple forests across Madagascar and elsewhere.

Although I detected lemurs in areas that both experienced low and high intensity logging, it is not clear whether lemurs breed in the more intensely disturbed forests or whether they depend on less disturbed parts of the forests for their reproduction. Research shows that for some species, the reproduction rate can exceed mortality in higher quality habitats (sources), but a local demographic deficit occurs in lower quality habitats (sinks), as the reproduction rate here is lower than mortality. In such situations, dispersal from sources may sustain populations in sinks and species may not persist in sinks without immigration from sources (Dias, 1996; Margules and Pressey, 2000). Research studies that identify such potential sources are important for conservation, in order to protect such forests accordingly and to prevent the species that need these sources from going extinct (Margules and Pressey, 2000).

A potential consequence of logging is a reduced resilience (Cole et al., 2014), which makes such forests more susceptible to further natural impacts, including extreme weather events (Dale et al., 2001). Thus, an improved understanding of the combined effects of human-induced and natural disturbances on the forest structure, composition, and functioning would be very relevant to predict and cope with the multiple disturbances that currently induce change in forests, worldwide.

Up to 90 percent of all tree species in tropical rainforests worldwide rely on frugivorous animals for their seed dispersal (Jordano, 1992). Such frugivores ingest whole seeds and defecate them at locations some distance from the parent tree (Razafindratsima et al., 2014). In some lemur species, germination of seeds is even accelerated by this process, and seeds become more viable after passing through the lemurs' digestive system (Dew and Wright, 1998). Particularly in disturbed habitats, the connectivity with pristine forests and the presence of seed dispersers is essential in forest regeneration, as these dispersers can initiate successional processes that

reinstate indigenous forest tree species (Holloway, 2000). Malagasy forests show a low richness in frugivore communities when compared to other tropical rainforests (Ganzhorn et al., 1997); of those, lemurs have the greatest biomass and species diversity. They are therefore considered to be the most important seed dispersers on the island (Hawkins and Goodman, 2003; Razafindratsima and Dunham, 2015; Wright 2005), as only a small number of bird species (Bollen et al., 2004), fruit bats (Cardiff and Jenkins, 2016), and possibly bush pigs (Andrianjaka and Droy, 2003) may otherwise fill this role. Hence, the regeneration of forests, with a complete set of primary forest tree species, may depend on the presence of seed-dispersing lemurs (Ganzhorn et al., 1999). As seed dispersal is often a major limitation to tree recruitment following human disturbances, the presence of lemurs is important in forest regeneration (Holl, 1999; Huffman et al., 1997; Razafindratsima et al., 2014; Wijdeven and Kuzee, 2000). However, lemurs are also excellent seed dispersers of invasive fruit tree species. They can therefore potentially facilitate the spread of such species, which consequently suppresses the regrowth of native tree species (Lowry et al., 1997). Although across Madagascar, invasive fruit tree species can be a valuable energy source for lemurs, monitoring and restraining the uncontrolled spread of invasive tree species may be needed as a measure to protect the original floristic diversity.

In the next chapter, I showed that the congeneric species *E. rubriventer* and *E. rufifrons* exhibit niche separation in several ways, which enables these closely related species to coexist (**chapter 3**). These results are based on diurnal observations. However, both species are known to be cathemeral, which means they can be active throughout the 24-hours cycle (Overdorff and Rasmussen, 1995). Differences in activity during the night could potentially enhance temporal niche separation (Curtis and Rasmussen, 2006). The lack of nocturnal data leads to an incomplete picture of the full activity cycle of these species and their potential niche separation during the night. Therefore, to fully understand activity patterns of both species, I suggest other researchers to include nocturnal activity patterns as well, to be able to evaluate the role of cathemeral behaviour in explaining coexistence patterns. Furthermore, I collected data during the wet season, while both species may exhibit different interaction rates and behaviour during the dry season (Curtis and Rasmussen, 2006). Collecting year-round data over several years would be needed for a more complete picture of species coexistence patterns.

In **chapter 4**, I recorded the microbial composition of multiple lemur populations across Madagascar, and show that season and geographic area drive the faecal microbiota of lemurs. The high fraction of poorly assigned bacterial microbial taxa reflects that Strepsirrhini primates have so far received little attention, harbouring a broad range of potentially novel bacterial species and genera that can be considered in future studies. Furthermore, I suggest that lemurs with access to

considerable quantities of sugar-rich fruits show lower faecal bacterial alpha diversity. Easy access to such food items—for example, to fruit tree plantations bordering forests, or to foods directly provided by humans—lowers the lemurs' activity levels. As such dietary changes increase the risk of diet-related health problems in humans, including obesity and diabetes (Bray, 2010; Marx, 2002), research on the consequences of such dietary changes for lemur health needs to be considered.

In addition to the faecal bacterial microbiota, I gave on overview of the parasite prevalence in lemurs (**chapter 5**). The parasites I detected were morphologically distinct on the genus level (Clough, 2010), which hampered identification to the species level. It is known that *Lemuricola* pinworms include eight different species in lemurs (Reviewed in Irwin & Raharison 2009), and the genus *Callistoura* includes two species (Chabaud and Petter, 1959). To unravel how these parasites co-evolve with and adapt to their hosts, how an altered environment changes their distribution, and if and to what extent these parasites influence the health of their hosts, it will be essential to genetically identify them to the species level.

Furthermore, the design of this study did not allow for repeated sampling of the same lemur individual over time, as I avoided resampling the same individual to ensure that all collected samples are from unique animals. Egg excretion of many GI parasites may not be constant over time (Villanúa et al., 2006) and, therefore, I could not detect infection intensity of these parasites. This puts forward the potential presence of false negatives within our dataset, leading to a possible underestimation of the real parasite prevalence. Future studies should take this into account, and should establish a false negative rate for their specific parasite and host species.

Next, primates are exposed to a much wider range of parasites and pathogens than the pinworms I investigated. Research indicates that anthropogenic habitat alteration is the most important variable associated with infectious disease outbreaks, including zoonoses (Dobson and Foufopoulos, 2001). Countries with poor living and health standards, like Madagascar, are especially vulnerable to such outbreaks. Pathogens and parasites of wildlife species, like lemurs, may be shared with humans and their domestic animals (Jones et al., 2008). Notorious examples of zoonoses resulting from increased contact between humans and wild primates are AIDS (Anderson, 1989; Wolfe et al., 2007) and Ebola (Daszak et al., 2000). Although most GI parasites have less dramatic impacts compared to such disastrous disease outbreaks, a variable number of parasite species related to the species found in lemurs can infect other hosts, including humans, domestic animals, and rodents. Nevertheless, no record of direct spill-over of such parasites has been documented in Madagascar to date (Hope et al., 2004; Howells et al., 2011; Kightlinger et al., 1995; Murata et al., 2002; Sleeman et al., 2000). However, rapidly shifting land use regimes in Madagascar increase the frequency of contact between humans and wildlife. Understanding in

greater detail how land-use changes influence the risk of zoonotic pathogen transmission among primates, humans, and domestic animals would be critical for designing intervention strategies to conserve lemurs and at the same time safeguard human and animal health.

Finally, this chapters' results suggest interactive effects between parasite infections and faecal bacterial microbiota composition. Recently, more studies identified interactions between microbiota and GI parasite species, as parasites can change environmental conditions prevailing in the intestine and thus influence the habitat of the bacterial microbiota (Koch and Schmid-Hempel, 2012). In turn, microbiota can influence colonisation of new parasite species, as well as within-host dynamics of parasites (Aivelo and Norberg, 2017; Hayes et al., 2010). Interactions between intestinal organisms can influence their ecology, but the specific relations between parasites and bacterial microbiota remain unclear (Reynolds et al., 2015). I therefore propose to consider the potential role of microbiome-parasite associations on the hosts' GI stability, health, and survival in future studies.

In **chapter 6**, I showed the loss of genetic diversity in an isolated lemur population. In Madagascar, more populations are becoming isolated (Irwin et al., 2010), and I recommend that future studies continue analysing *DRB* polymorphism as a proxy for immunocompetence. Our newly developed primer set can now be used, specifically in studies on *Eulemurs*, but very likely on other non-human primates, as well. In addition to the MHC class II genes, other genes can influence parasite and pathogen resistance (Acevedo-Whitehouse and Cunningham, 2006). However, I focussed solely on MHC genes, as their function and evolution is relatively well described compared to other genes involved in immune responses, and because they are the most polymorphic genes known in vertebrates (Bernatchez and Landry, 2003; Sommer, 2005; Piertney and Oliver, 2006). The class II genes are physically linked, and alleles on these genes are in strong linkage disequilibrium (Marsh et al., 1999). Therefore, *DRB* gene diversity patterns can be a good indicator for the genetic variation in other class II genes, and even for other less closely linked MHC genes (Kelley et al., 2005). Quantifying MHC II *DRB* diversity can assess a species' ability to respond to infections (Unanue et al., 2016), which could arise due to ecological changes induced by changing climatic conditions, habitat loss, and forests fragmentation. Especially for small and isolated populations, quantifying and monitoring MHC II *DRB* variation is recommended to establish successful conservation plans that mitigate the loss of genetic diversity in lemurs. Furthermore, investigating the association between MHC II *DRB* variation and disease resistance or other fitness parameters (such as body condition, life span, reproduction success, social status, microbiome, and disease patterns) would increase our understanding of the role of diversity in MHC genes in the health status of populations. Based on my

thesis' results, I recommend quantifying and monitoring variation in MHC II *DRB* and other genetic markers of immunocompetence and resistance against parasites and pathogens, in order to establish successful conservation plans that mitigate loss of genetic diversity in lemurs, especially in small and isolated populations.

The effect patterns of natural and anthropogenic disturbances, and their impacts on the health of lemurs, likely apply to other wild mammal communities elsewhere in the world, as well. However, extrapolation to other primate taxa might be impeded by the fact that primates are extremely diverse with regard to their environment, functional morphology, and their social systems (Rowe and Myers, 2016). Nevertheless, results of this thesis provide a good basis for further investigations that aim to unravel the impact anthropogenic and natural disturbances on primate populations. I propose that comparative studies should be conducted in other primate and wildlife species to determine the generality of these findings.

OTHER CHALLENGES AHEAD: CLIMATE CHANGE

Madagascar and the island's wildlife face multiple other challenges that could not be addressed in this thesis. To mention a few, political instability, invasive species, locust outbreaks, infectious diseases, poor governance, corruption, and extreme poverty could all form incentives to cut down forests to survive. In addition, climate changes and extreme weather events, leading to droughts or floods, form major challenges for local communities as well as Malagasy forests (Walther et al., 2002). I presume that the synergistic interaction of both human-induced and natural impacts is the largest threat to the remaining Malagasy forests. Anthropogenic environmental perturbations impact forest ecology and resilience, which determines the degree of damage from natural disturbance regimes that forests can withstand (Chazdon, 2003; Smith et al., 2009), including an increased frequency and severity of rare weather events due to climate change (Webster et al., 2005).

In Madagascar, clear evidence exists that climate shifts are currently occurring, leading to changes in precipitation patterns and increased temperatures (Tadross et al., 2008). Temperature and precipitation are forecasted to increase throughout the island, except for the dry southern region, where droughts will become more profound (Hannah et al., 2008). Such droughts will especially be severe in an El Niño year, a phenomenon that is becoming more frequent (Fedorov and Philander, 2000). Also, the intensity of cyclones is expected to increase (Tadross et al., 2008). Such climatic changes have already begun to impact lemur populations and their reproductive success (Hannah et al., 2008; Raxworthy et al., 2008). More broadly, these changing environmental conditions may impact the health of multiple wildlife species, the epidemiological dynamics of diseases, and host-parasite associations (Barrett et al., 2013; Duncan et al., 2011). Over the coming decades, lemur

populations have to adapt quickly, or they will be forced to shift their ranges into new, suitable habitat in response to future climate changes (Willis and Birks, 2006). However, opportunities for range shifts are limited, as forests are scarce, and novel ecological interactions form another challenge ahead.

LEMUR CONSERVATION

Over the past decades, the unique biodiversity in Madagascar—and specifically lemurs as flagship species—has built public awareness (Rakotomamonjy et al., 2015; Rowe and Myers, 2016; Wright et al., 2014). The status of lemurs has been especially effective in promoting fund raising for conservation efforts. By protecting lemurs and other charismatic species, countless other species that rely on the same forests can simultaneously be protected (Sanderson et al., 2002). Lemurs need relatively large and diverse areas that encompass the resource requirements of many other plant and animal species. Conservation of iconic lemur species will therefore not only lead to the conservation of other sympatric lemur species, but presumably also to the conservation of the forest structure, composition, and ecological functions in different habitat types. Therefore, by conserving lemurs, the conservation needs of many other species can be secured (Sanderson et al., 2002).

At present, Madagascar receives the largest amount of funds for research and preserving or protecting the environment, natural resources, and biodiversity compared to any other part of Africa (Neudert et al., 2017). How should these funds be allocated to guarantee successful conservation, and how can conservation incentives be aligned with the norms and values of local communities? Currently, 53 national parks, managed by Madagascar National Parks (MNP), and several special reserves are located throughout Madagascar, including two world heritage sites. These protected areas comprise about 37,000 km^2, 6.3 percent of the total area of the island (Jolly et al., 2016). In forest management, the value of research is sometimes questioned (Belter, 2014), and integrating multiple forest management aims—including harvesting forest products, conserving wildlife habitat and biodiversity, and tourism—can be complicated (Solomon and Dereje, 2015). I will now address these issues in more detail.

THE ROLE OF RESEARCH

To generate achievable management strategies and to develop targets with regard to successful lemur conservation and reforestation, there is a clear need for large-scale and long-term monitoring studies (e.g., Franklin 1989). Extensive studies on ecosystem functioning and the impacts of habitat alterations will provide a more complete picture that can be integrated in conservation plans and decisions, and will improve environmental management (Bennett, 2016; Walsh et al., 2015). This is

essential for identifying species and areas that are most at risk and need urgent attention, and in deciding which conservation actions should be prioritised and implemented (Petrovan et al., 2018). Furthermore, research on the habitat requirements of wildlife species is needed to develop effective restoration efforts, and to identify and protect the focal landscapes, physical elements, and resources that are necessary to ensure the survival of target populations (Sanderson et al., 2002).

Although conservation practitioners may value scientific information, they need access to it as well (Sunderland et al., 2018); for example, through the availability of open access publications (Fuller et al., 2014). Making clear and concise summaries and implications of research findings can make it more likely that scientific results are used in conservation plans, as such summaries reduce the time and skills practitioners need to comprehend the information (Petrovan et al., 2018; Walsh et al., 2015). To be effective, scientific research should be considered along with the specific local situation and practical considerations, such as cost accessibility (Petrovan et al., 2018). Thus, evidence-based conservation can advance wildlife conservation in managing the major challenges we face globally, but it is important to consider the means of communicating scientific output to local conservation management.

Furthermore, the physical presence of researchers in the forest is an effective deterrent against illegal activities (Laurance, 2013). Researchers also involve many local people and provide local jobs. Locals are a very valuable source of knowledge and are generally indispensable as research guides or technicians. They are often involved in long-term population surveys, habitat assessments, and forest monitoring, and (after receiving appropriate training) even continue data collection independently in some cases (Sheil and Lawrence, 2004). In addition, local communities can be involved in many other research-associated activities. For example, they help build research camps and create trails, porters help to transport camp equipment and provisions to remote areas, and local markets benefit from the sales of food and water that research teams need during their expedition. Furthermore, each researcher also supports a local Malagasy student during fieldwork. This student could have a major role in raising awareness and support for conservation in Madagascar, and may potentially have a large impact as a future local conservationist (Marcus, 2001).

This thesis describes many challenges lemurs in Madagascar face today and although conservation-oriented research is essential to establish successful conservation management (Sutherland et al., 2004), forests and wildlife cannot be saved through following and observing animals, alone. Direct conservation actions, including minimising rainforest loss, reforestation, establishing buffer zones, and creating corridors, are necessary to prevent forests from disappearing and species from going extinct (Newbold et al., 2014). Thus—although observing lemur behaviour, monitoring species and habitat trends, and assessments of the conservation status of

species are all important—we, as researchers, should not let species go extinct while observing them, and should directly translate research outcomes into practical conservation actions to contribute to the survival of the species we study.

FOREST PROTECTION

Human interference may be needed in forest recovery and in mitigating effects of disturbance. Such conservation incentives include the establishment of protected areas, thereby preventing further forests losses. People can also establish wildlife corridors, reforested areas (Hale and Lamb, 1997; Janzen, 1988; Kaiser, 2001), and fire-breaks to prevent uncontrolled forest fires (Nepstad et al., 2001). Based on scientific field studies, optimal reforestation programmes can increase the number of trees that wildlife species need for their survival, and can optimally link forests fragments via corridors (Pardini et al., 2005). Tree nurseries can be set up, including both native Malagasy tree species and commercial fruit trees that provide food for local communities (Gould and Andrianomena, 2015). Although there is a lot of critique on community-based conservation (Dressler et al., 2010), community-based conservation incentives can provide alternative livelihoods for at least part of the community. However, this will not solve all systemic issues present, such as poverty, and the need of land for housing, agriculture, and forest products remain (Buckley, 2010). There obviously is a trade-off between food production and nature conservation (Andrianirina et al., 2011). In Madagascar, the percentage of agricultural land has increased from 62.7 percent in 1995 to 71.2 percent in 2015, and forest cover has decreased from 23.0 percent to 21.4 percent over the same period (Boysen et al., 2017). These trends emphasise the need for actions in order to cease further deforestation. Two strategies exist that reconcile the need for forest conservation and the pressures of agriculture: land-sharing and land-sparing.

In a land-sharing approach, forest protection is combined with forestry or cultivation of specific crops, while the natural forests are maintained as intact as possible (Phalan et al., 2011). For example, reduced-impact logging techniques can be applied in some Malagasy forests, with restrictions on the size and number of trees that could be harvested, and limitations on the use of heavy machines to avoid soil destruction (Davis, 2000; Forshed et al., 2006). However, this may not be economical, and wildlife species would continue being exposed to the stress induced by logging activities (Pearce et al., 2003). Next to forestry, agriculture could take place in forests. For most crops, unfortunately, the agricultural yields per unit area would decrease when they are grown in forests. However, land-sharing can be successful for several crops and spice plants that thrive in the forests' understory, without impairing forest functioning. Vanilla orchids, peppers, and multiple spices are among many crop examples that thrive in full shade. Also, honey can be produced in many natural areas,

with only minimal impact to forests (Bradbear, 2009). The production of such goods therefore results in benefits for both the local economy as well as forest protection. I propose that the implementation of such specific initiatives across Madagascar can generate alternative incomes to local communities, thereby reducing the pressure on the natural environment. To meet the production demands with land-sharing, a relatively large area would be needed. This exposes more wildlife species to agricultural activities, and may lead to stress experienced by living in human-modified landscapes. Therefore, the alternative method—land-sparing—will most likely be necessary, to produce food supplies sufficient enough to sustain the Malagasy community.

Land-sparing is a strategy that clearly separates natural areas from agricultural fields. In this approach, wildlife habitats are 'spared' from conversion into agricultural land, and high-yield farming of crops is stimulated outside the forests (Green et al., 2005; Phalan et al., 2011). By applying fertilisers and pesticides, the yield is substantially increased, and the area under cultivation is minimised. For example, the System of Rice Intensification (SRI) is highly successful in enlarging rice yields, while reducing the required seeds and water (Satyanarayana, 2004). Here, the productivity of irrigated rice is increased by changing plant density, improving soil conditions for root and plant establishment and development, and enriching the fields with organic matter that contains essential plant nutrients. Technical studies on increasing production rates of local crops, restoring water and nutrient conditions of the soil, and other means to increase the efficiency of agriculture are needed to reduce the land required to sustain the local communities. I consider combining land-sharing and land-sparing as a promising strategy to lower the area that is needed to meet the local food demands, in combination with protecting wildlife habitats.

In Madagascar, people have a strong connection with nature, and forests play an eminent role in their culture (Desbureaux and Brimont, 2015; Neugarten et al., 2016). For example, they believe that their ancestors are manifested in many plant and animal species, and that parts of the forests are sacred and function as residence for ancestral spirits. Like in most other underdeveloped economies, local communities depend heavily on the resources provided by the environment (Huang and Pray, 2002). They extract many primary resources from forests for their subsistence, including forest products such as fruits, honey, yams, bush meat, and many species of medicinal plants (Shackleton et al. 2011). Assigning nature as a protected area can have negative impacts on communities, especially on those that are located in close proximity to such forests. After the inauguration of a national park, local people have restricted or no access to the forest, leading to major consequences for poor rural household, including impacts on their cultural and religious values (Scales, 2014).

The perception of nature conservation differs throughout Madagascar and

depends on multiple factors, including a persons' tribe, residential area, age, level of education, primary occupation, indigenousness, frequency of contact with foreigners, proximity to conservation areas, and the economic impact of conservation on the local households. Protected areas can only be effective if the local community complies with the new rules. People first have to see the value of nature and the need for conservation (Mascia et al., 2014). Therefore, extensive communication between locals and conservationists is eminent to create awareness for the benefits of conservation, and the potential of conservation to provide alternative livelihoods. This communication is needed to establish local understanding, acceptance, and support for conservation actions (Hanson et al., 2012; Scales, 2014).

People in Madagascar use most of their land for their traditional forms of land use, especially slash-and-burn rice farming. Rice has been cultivated for centuries and is the main subsistence crop in Madagascar (Crowther et al., 2016). Eating rice, preferably 'mountains of rice', and preferably three times a day, is the rule in the culture of the Madagascan community, and rice forms the primary source of income for many local households (Harvey et al., 2014). Giving technical advice on sustainable resource use, agriculture, and on techniques to increase the harvest are important to reduce the amount of land and energy resources used. To give an example, initiatives exist that introduce alternative cooking methods to reduce the use of charcoal and firewood. Traditionally, Malagasy cook their rice over open fires, which is not fuel-efficient and—when performed indoors—it strongly increases the risk of respiratory diseases. New techniques, like fuel-efficient rocket stoves or solar cooking as replacements for the inefficient open fires, lower resource use and smoke development (Urmee and Gyamfi, 2014). Nevertheless: as with eating rice, cooking on open fires is deeply rooted in Malagasy culture, which makes it a challenge to convince the community to adopt such new techniques. For all changes in peoples' daily behaviour, it is important to invest in mutual understanding and trust to implement such new practices. Involving local stakeholders, elders, and other respected residents of the area in the creation of nature conservation plans and in implementing new techniques often increases the support of the whole community. Such technical changes can support both the subsistence of local people, as well as the forests that people use to survive.

ECOTOURISM AS A SOLUTION?

International tourism has been growing over the last decades. Foreign visitors are mainly attracted by Madagascar's high biodiversity and endemic flora and fauna, with the charismatic lemurs as the predominant focus (Peypoch et al., 2012). International tourism is increasingly important for the economy of Madagascar, with an ongoing rise in the total contribution to the GDP, which has increased from 5.8 percent in 1997 to 13.7 percent in 2016. The total employment in the tourism sector was 11.4 percent,

and tourists generated 17.9 percent of the total exports in 2016 (WTTC, 2017). Basically all tourists visit at least one of the national parks, protected areas, or special reserves across the island. In Madagascar, ecotourism, defined as "responsible travel to natural areas that conserves the environment, sustains the well-being of the local people, and involves interpretation and education", makes up the largest segment of the tourism sector (Christie et al., 2003). Tourists usually only visit a minor part of a protected area but generate substantial revenue to ensure the protection of a complete national park. As ecotourism can generate employment possibilities for local people with limited environmental impacts, it is one of the prime examples where conservation of forest leads to important economic benefits (Chomitz et al., 2005; Norton-Griffiths and Southey, 1995).

Hereby, ecotourism creates a link between alternative and often improved livelihoods and the conservation of natural areas (Kiss, 2004; Krüger, 2005). Most ecotourists are usually willing to pay directly for forest preservation in the form of park entrance fees and the hiring of local guides. The generated revenue can be allocated to the continuation of park protection, park expansion and maintenance, as well as the necessary training of guides (Walpole et al., 2001). Many new employment opportunities arise with the establishment of protected areas, including: working as wildlife guide, spotter, or protector of park boundaries; employee in service areas like restaurants, hotel accommodations, or as a driver; manufacturer or salesman of local arts and handicrafts; or as worker in park maintenance, including the construction of campsites, buildings, and infrastructure. People are also needed for surveillance within a protected area to keep watch for activities that are damaging the ecosystem and to report suspicious activities. Many jobs are suitable for both youth and older people, as well as for both men and women, and they enhance economic independence and security. When ecotourism generates more income than activities like slash-and-burn agriculture or commercial logging, protecting forests therefore delivers greater rewards than resource extraction (Buckley, 2010; Burns and Sofield, 2001). Hereby, economic benefits exceed the opportunity costs of other forest activities, and the losses people experience when their access to forests is ceased are compensated for (Xiang et al., 2011). This can be a major incentive for local communities to support conservation and to protect forests and wildlife, instead of unsustainably extracting natural resources from forests (Tisdell and Wilson, 2012).

Next to changes in livelihoods and behaviour in local communities, ecotourism has the potential to influence the behaviour of tourists that are visiting the forest. Ecotourism brings visitors in closer contact with nature, thereby increasing public appreciation of the environment and awareness of the threats natural ecosystems are facing. This may stimulate sustainable behaviour in their daily lives, and may raise support for conservation in terms of funding (Cárdenas et al., 2015). In this way, the

effects of ecotourism on the preservation of the environment can be both local as well as global.

Besides the mentioned benefits of ecotourism, there are also associated environmental costs (Buckley, 2012, 2010). By both international as well as local transport, tourism contribute to pollution of atmosphere, oceans, and fresh water. Flight emissions contribute 4.6 percent to the total anthropogenic greenhouse gas emissions and are expected to continue in the coming decades (Gössling et al., 2007). In addition, tourists consume an enormous quantity of goods and services and, therefore, sustainable and smart production is needed to limit impacts on the environment. Wildlife tourism can also have other unwanted consequences, including illegal capture of wild animals for up-close encounters or exhibition to tourists (de Winter, *personal observation*). Furthermore, the term 'ecotourism' is regularly misused by tour operators to attract customers, so operators should regularly be inspected to ensure they are only causing relatively-low impacts to the environment. Another problem is that the economic benefits generated by ecotourism are often captured by only a few stakeholders, such as wealthier villagers, large tour operators, and taxi companies (WTTC, 2017). This leads to an uneven spread of benefits within local communities, economic inequity, and potential conflicts. It is therefore important to translate the values generated by conservation into actual local benefits, to motivate complete local communities to obey the rules and preserve the natural resources. Here, the income generated by tourism has to be sufficient to sustain households year-round, in order to cause complete economic shifts away from illegal activities, especially during low tourism seasons (Balmford and Whitten, 2003; Hanson et al., 2012). However, national parks and reserves in Madagascar face many challenges in combining the sometimes-conflicting demands of biodiversity conservation and nature tourism. I will discuss a few of these main conflicts here.

First, there is a difficulty in aligning conserving 'authentic' forests and cultures with the presence and demands of tourists (Urry, 1995). For up-close wildlife encounters, wildlife has to be habituated to human presence. Tourism, by itself, can induce changes in 'authentic' local livelihoods and cultures (Ramkissoon, 2015). In addition, the presence of tourists in forests can lead to environmental degradation: by waste disposal, treading down vegetation (Mason et al., 2015), and by the elaboration of infrastructure, including roads and forest trails, increased car traffic, and forest fragmentation (Huhta and Sulkava, 2014). People want to see aesthetic, pristine sites, but domination by tourists, the creation of trails, and habituation of animals contrast with the pristine forests people urge to see.

Second, the presence of humans in forests and their close proximity to wildlife can induce both acute and chronic stress to animals (Maréchal et al., 2011; Walker et al., 2006). Human presence can impact the behaviour, ranging pattern, and diet of

wildlife species, especially when species are not yet habituated (Fuentes, 2012; Remis, 2000). For example, primates can show increased vigilance, decreased feeding in the presences of tourists, and avoidance behaviour, like fleeing from humans or moving higher up in the canopy (reviewed in Maréchal et al. 2011). As discussed in this synthesis, feeding by humans often leads to dietary changes. This can elevate stress levels, triggered by the close proximity to humans, or because of encounters with conspecifics that are competing over the food provided (Maréchal et al. 2011, de Winter, *personal observation*). The often sugar-rich diet supplements provided by tourists can cause serious health problems for these animals, in the long run. It can make animals dependant on such provisions, and can increase the risk for diet-related health problems like obesity and diabetes (Junge et al., 2009; Kavanagh et al., 2013). Research that involves the monitoring of stress and health parameters, as described in this thesis, in areas with increased tourism, should lead to the establishment of regulations that minimise chronic stress. This is necessary to ensure the health of wildlife animals that, in turn, are of interest to tourists.

Third, increased human presence in forests can change interaction patterns between wildlife and humans, domestic animals, and several species of rodents (Gillespie et al., 2005; Nunn and Altizer, 2006; Sorci et al., 1997). Close contact between tourists and wildlife creates potential transmission risks of pathogens from humans to wildlife, as well as diseases from wildlife to humans, i.e., zoonoses (Hudson et al., 2006; Junge et al., 2011; Koprowski, 2005; Rickart et al., 2011). This is a particular concern for lemurs, as primates are relatively more vulnerable to many human diseases and vice versa when compared to more distantly related wildlife species (Brearley et al., 2013). Infectious diseases are emerging worldwide at an accelerated rate in both human and animal populations (Daszak et al., 2000; Morse, 1995), and environmental and land use changes are among the main critical factors influencing this disease emergence (Institute of Medicine, 1992), especially in poor countries with limited health care. Therefore, potential zoonoses and transmission of other diseases are a major concern and should be monitored (Lafferty and Gerber, 2002).

Economic benefits created by tourism should be shared within local communities. Furthermore, opportunities for as many locals as possible to play a role in tourism should be stimulated (Neudert et al., 2017). This is a way to compensate for the direct and indirect negative impacts on the local community, thereby increasing peoples' motivation to accept the new restrictions. Establishing this strong link between nature conservation initiatives and poverty alleviation of the local communities is key in convincing locals to accept conservation initiatives (Ferraro et al., 2015).

In addition to generating income, utilising the revenue earned via tourism to provide other benefits to the villages that surround protected areas, will likely add to

local acceptance. Such opportunities may include establishing facilities for education, community centres, and sanitation. Organising activities, such as conservation events and festivals, can help integrate conservation into the culture of local communities. Such community events, like yearly sport contests for lemurs (e.g., the 'Maki Run') and celebrations (e.g., the 'World Lemur Festival' and 'World Lemur Day'), can unite and involve people in conservation and can change their perception and value of their countries' nature. In all cases, when conserving wildlife habitats, we should take the costs and benefits that local communities experience into account; we need to address social needs, and make compromises to ensure peoples' welfare, when required.

I recommend extensive communication between locals and conservationists to create local support of conservation initiatives. When local villages benefit from ecotourism activities in the forests they live adjacent to, this creates social pressure, thereby reinforcing the new regulations and reducing corruption. The presence of local guides, spotters, and tourists in the forests, can strongly reduce illegal activities, as offenders are much more afraid of being caught (see also Sommerville et al., 2010). If people in communities are involved in conservation, and benefit from protecting the forests in which many endangered species reside, they are more inclined to ensure the forest's safety.

People across the world are willing to travel great distances and pay significant amounts of money to have rewarding personal experiences from encounters with nature and wildlife species. This generates a major source of income for countries harbouring such natural systems, thereby creating incentives to conserve the remaining vegetation, and to promote sustainable forest utilisation as an alternative to timber exploitation and poaching for the wildlife, bush meat, and pet trade. However, the growth of tourism leads to changes in human-environmental relations that should be observed critically. The number and size of protected areas in Madagascar has increased over recent decades, and this trend is likely to continue. Ecotourism, when carried out in a sustainable manner, can have a crucial role in ensuring the effective management of rainforest ecosystems, can significantly contribute towards preserving biodiversity, and can be effective in poverty alleviation (Buckley, 2012).

For that reason, ecotourism needs to be managed adequately, and regulations to minimise the impacts of visitors on the environment and animal habitats are needed. For example, there should be a daily maximum limit of tourists that are allowed within a protected area, and close contact with and feeding of animals should be prohibited to minimise stress and behavioural changes in wildlife and to lower the risk disease transmission. Tourists should maintain a minimum distance, waste removal should be encouraged, and noise levels should be kept down. Such measures can reduce the impacts of tourism on wildlife and the ecosystem, contribute to maintaining the integrity of a forest site, and I personally believe that these

restrictions even add to the experience of visiting a natural area.

To achieve the described conservation incentives, investment in education is key (Jacobson et al., 2015). Some locals have already acquired part of the needed knowledge and skills from their previous work in the forests, e.g., poaching and logging, which can now be used in guiding tourists or researchers. The community needs training to fulfil their new jobs, for example as a research guide or cook. Part of the education could be the organisation of forest visits by local communities, school children, and university students to make them realise the importance of wildlife and the need to conserve nature. In this way, we can hopefully make more people care about their natural environment, thereby stimulating locals to shift from being forest exploiters to forest protectors.

A SAFE HARBOUR?

The work described in this thesis forms an integrated approach, linking the impacts of anthropogenic and natural challenges on both the occurrence and multiple health aspects of lemurs on different geographic scales. I non-invasively identified such challenges on both forest structure and composition as well as the presence, behaviour, and health of several threatened lemur species across Madagascar. I also considered the future of biodiversity conservation in Madagascar. This thesis demonstrates that although anthropogenic disturbances can alter forest structure and composition, regenerating forests can have considerable conservation potential as lemur habitat. Such disturbances can also create landscape heterogeneity that facilitates coexistence of congeneric lemur species. Disturbances may exert stress on lemurs and can affect their overall condition and immunocompetence. Anthropogenic disturbances and biogeographic differences impact the lemurs' microbiome, GI parasite levels, and MHC II diversity. Such changes may impair the capacity of lemurs to withstand additional pressures, and thereby their survival potential. Monitoring the presence, behaviour, and health parameters of animals is critical to detect early warning signs of stress or an impaired health.

On natural, floating rafts of vegetation, a lemur ancestor migrated from mainland Africa across the Mozambique channel about 60 million years ago. The species arrived on a large 'raft' called Madagascar and found refuge on what seems to be a permanent, safe harbour, covered with lush rainforests with plenty of fruit, limited predation, and no competition exerted by higher primates. Over millions of years, a combination of climatic and physical geographic isolation, and competition between sympatric lemur species, led to the specialisation of lemurs into multiple distinct ecological niches. Adaptive radiation has resulted in more than one hundred different species that occupy practically all vegetation and climate zones across the island. Nearly all species function as plant pollinators or seed dispersers in the diverse

ecosystems. In this apparent paradise, many natural challenges, including flooding, multiple volcanic eruptions, and climatic changes, have challenged lemurs over time. Human history on this island began about 2000 years ago, leading to human-mediated deforestation and land-use changes. As a result, only 10 to 20 percent of the original forest cover currently remains. Lemurs constitute an important part of the global biodiversity, forming a unique assemblage comprising more than 20 percent of all primate species that are known today. However, the ongoing decline and degradation of lemur habitat, in combination with a changing climate, threatens the future of lemurs and other wildlife species. Some lemurs can only survive in undisturbed forests, whereas others prefer selectively-logged forests. But very few can live without forests. This puts into question the stability of the once resilient raft: how long can lemurs, and the unparalleled species richness that has evolved in Madagascar, use this raft as a safe home?

On the one hand, there are still breath-taking forests in Madagascar; forests that house a variety of endemic flora and fauna. On the other hand, the amount of forest today is less than at any time since Madagascar was first inhabited by humans. Despite significant conservation efforts, habitat loss and degradation in Madagascar has continued (Herrera, 2017). Although some species may be able to cope with land-use changes, the ongoing deforestation will not be sustainable in the long run. I am convinced that conservation organisations, researchers, as well as tourists can build the capacity necessary to advance conservation and research, thereby contributing to the long-term survival of the remaining populations of lemurs. Community-based conservation actions that contribute to the protection of Madagascar's sensitive environment will be essential. Such actions include (1) expanding protected areas, stimulating reforestation, and increasing forest connectivity; (2) preventing excessive clearance of forests by implementing alternatives, including ecotourism and more efficient agriculture techniques; and (3) setting up collaborative, multidisciplinary, and long-term monitoring programmes of lemur health parameters. To implement such actions, communication and collaboration between local authorities, conservationists, and researchers will be a critical component in future lemur conservation. In a country where arable land is sparse and people are very poor, the fate of lemurs as well as human inhabitants should be considered together when addressing the major challenge we face globally: meeting human needs while sustaining wildlife. Proper conservation actions, based on the results of this thesis and further investigations on the impact of natural or human-induced habitat alterations on primate populations, can help to ensure the long-term viability of threatened species. Thereby we can keep the raft called Madagascar, including its unique flora and fauna, afloat.

Box 7.1 Overview of the main questions and answers of this thesis per chapter.
A more complete description of these questions and answers can be found in these associated chapters.

Chapter 2
Can the impact of past logging still be discerned in forest structural characteristics?
Despite a recovery period of nearly 30 years, our results from a rainforest in Madagascar show that the impact of past logging can still be discerned in forest structural characteristics and result in smaller trees, higher stem densities, lower heterogeneity in tree height and diameter, as well as a lower diversity in tree species and families.

Have previously logged forests recovered into functional lemur habitat?
The forests that experienced intense logging in the past seem to have recovered from a functional perspective into suitable lemur habitat, as lemur encounter rates and cluster sizes were fairly similar in such forests when compared to forests that experienced limited logging intensities.

Chapter 3
How can the coexistence of the congeneric lemur species *Eulemur rufifrons* and *E. rubriventer* be explained?
Large-scale spatial segregation into different areas within a heterogeneous environment, caused by previous logging, in combination with niche differentiation, facilitates the coexistence of these congeneric species.

Box 7.1 continued

Chapter 4

To what extent does location, species, sex, and age influence the faecal bacterial microbiota composition in lemurs?

Occupancy had the strongest influence on intestinal microbiota of congeneric lemur species. The influence of lemur species was minor, and there was no influence of sex and age detected.

Chapter 5

How do geographic location, seasonality, and anthropogenic disturbances influence parasite infections and faecal bacterial microbiota composition in lemurs?

Infections with nematodes of the genera *Callistoura* and *Lemuricola* occurred in all lemur populations. Seasonality significantly contributed to the observed variation in microbiota composition, especially in the dry deciduous forests. Microbial richness and *Lemuricola* spp. infection prevalence were highest in a previously intensely logged site, while *Callistoura* spp. showed no such pattern.

Is there an interactive effect between GI parasites and microbiota?

I observed significant correlations between the presence of gastrointestinal parasites and bacterial microbiota.

Chapter 6

What is the variability of immunologically important genes (i.e., MHC class II *DRB*) among geographically separated *Eulemur* species?

The *DRB* genes of all three brown lemur species in our study express higher rates of non-synonymous substitutions in the antigen-binding sites, leading to different amino acid sequences. This indicates the presence of balancing selection, leading to high levels of *DRB* diversity and polymorphism.

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SUMMARIES

ENGLISH

Lemurs on a Sinking Raft?

The ballast of anthropogenic disturbances

Like all of the earth's natural ecosystems, tropical forests are influenced by a wide range of anthropogenic and natural impacts. Because of ongoing loss of these forests, many species have become vulnerable and population densities and distributions of many species are in decline. Human interferences with the environment are not limited to mainland forests. Human discovery of numerous remote islands has led to local landscape modifications and biotic adjustments. Due to their isolation, these islands are often home to a diverse array of ecosystems that host endemic flora and fauna.

Despite its proximity, the island Madagascar has a very different biotic and human history when compared to mainland Africa. Continental drift led to the island's isolation, and since then, a number of distinct biomes have developed. Vertebrate colonisation of Madagascar most likely occurred via dispersal on floating rafts of vegetation across the ocean millions of years ago. Most likely by floating on such rafts, a lemur ancestor arrived on the enormous 'raft' called Madagascar. Lemurs, a clade of Strepsirrhine primates, exhibit a number of traits that are considered ancestral for the order Primates. Over millions of years, lemurs have faced extreme differences in climate and geology across the island as well as across seasons. In combination with a lack of competition from real apes and limited predation pressure, these differences contributed to their radiation into more than one hundred different species.

Given that lemur survival is currently threatened by intense anthropogenic pressure and additional natural impacts, examining the responses of these endemic primates to several forms of ecological stress is an urgent priority. Historically, natural environmental alternations on Madagascar include abrupt climatic changes, extreme weather events, periodic fires, ocean dynamics, volcanic eruptions, and shoreline changes. Furthermore, major anthropogenic landscape modifications and ecological changes have occurred since human arrival in Madagascar about 2000 years ago, with far-reaching consequences for the indigenous species present. Anthropogenic disturbances and biogeographic differences impact the lemurs' microbiome, gastrointestinal parasite levels, and major histocompatibility complex II (MHC II) diversity. Such changes may reduce the capacity of lemurs to withstand additional pressures, and thereby their survival potential. Monitoring the presence, behaviour,

and health parameters of lemurs is therefore important to detect early warning signs of stress or impaired health. I aim to provide an overview of the complex relationships between multiple anthropogenic and natural impacts and the presence, behaviour, and health of lemurs across the island Madagascar. To my knowledge, this study will be the first in this integrated perspective, linking the impacts of anthropogenic disturbances and environmental challenges to the occurrence and relevant health parameters of true lemur species (genus *Eulemur*) on different geographic scales. The overriding expectations of this thesis are 1) that anthropogenic disturbances (i.e., selective logging) alter lemur encounter rates and facilitate the coexistence of closely related species, and that 2) these disturbances, as well as natural challenges, influence the lemurs' microbiota composition, gastrointestinal parasite levels, and MHC II *DRB* diversity.

My thesis consists of two parts: first, I link the effect of anthropogenic disturbances to forest structural changes, lemur encounter rates, and lemur coexistence. Second, I explore the physiological responses of lemurs to anthropogenic disturbances and environmental challenges. **Chapter 1** forms a general introduction that sets the scene by presenting the theoretical framework of this thesis. It further provides information on the biological background of the focal species and geographic locations. **Chapter 2** describes the impacts of anthropogenic disturbances on forest structure and composition, as well as encounter rates and group sizes of all diurnal species within a lemur community. Although the disturbed forests have not recovered to pre-logging conditions, they seem to have recovered from a functional perspective into suitable lemur habitat, as lemur encounter rates and cluster sizes are similar in disturbed and less-disturbed sites. The results of this chapter suggest that there is considerable potential for previously logged, regenerating forests to support lemur communities. In **chapter 3**, I focus on the role of human disturbances, niche differentiation, and direct competition in the stable coexistence of two congeneric and sympatric lemur species that share many ecological characteristics. The chapter contains the results of a quantitative behavioural study of habitat selection and direct competition between the species. As was found in chapter 2, different disturbance histories can create spatial heterogeneity in forests, which likely facilitates the large-scale spatial segregation of the two species. This segregation, in combination with species differentiation in resource use, time, and space and agonistic interactions, facilitates the coexistence of congeneric lemur species.

Next, **chapter 4** focuses on the faecal microbial composition in different lemur species occupying varying habitats. Here, I show that biogeographic differences and associated dietary compositions, rather than host species, age, or sex, are the main drivers of the lemurs' microbiota composition. In **chapter 5**, I provide an analysis of

environmental variation and anthropogenic disturbances on the faecal bacterial microbiota and gastrointestinal parasites in lemur species across Madagascar. Infections with nematode species of the genera *Callistoura* and *Lemuricola* occurred in all lemur populations, and *Lemuricola* spp. infections were elevated in previously logged sites. Biogeographic variation, seasonality, and forest disturbances are drivers of the faecal bacterial microbiota composition. I also report an interactive effect between gastrointestinal parasites and bacterial microbiota composition. In **chapter 6**, I perform a comparative study on the allelic variation (i.e., variants of a gene) within the MHC II, which is considered as an important proxy for lemur health. From non-invasively collected faecal and hair samples from multiple *Eulemur* species in different geographic areas, I successfully genotyped elongated DNA fragments with a newly developed DNA primer set, amplifying nearly all polymorphic codons, which code for amino acids, of the antigen-binding site. This DNA primer set can be used in future immune genetic health assessments of *Eulemur* species and likely of other non-human primates as well. I detected novel MHC II *DRB* alleles with a high level of sequence and functional polymorphism, which could be the result of different pathogen-driven adaptive selection pressures on lemur populations living in different geographic areas. I also found supporting evidence for the link between parasite presence and MHC II diversity. Furthermore, the number of unique alleles present in a lemur population living on a small island was much lower than in mainland lemur populations. Many forest fragments parallel islands in terms of area and isolation. Therefore, conservation plans that mitigate loss of genetic diversity in lemurs need to be established, especially in small and isolated forest fragments. In **chapter 7**, I synthesise the results of this thesis and discuss other challenges that biodiversity conservation in Madagascar is facing in the near future. The ongoing decline and degradation of lemur habitat, in combination with a changing climate, threaten the future of lemurs and other wildlife species. This puts into question the stability of the once stable 'raft' called Madagascar. In order to overcome the many threats species are facing, successful management of nature has never been more important.

In this thesis, I found that, although anthropogenic disturbances can alter forest structure and composition, regenerating forests can have considerable conservation potential as lemur habitat. Different logging intensities can create landscape heterogeneity that facilitates coexistence of congeneric lemur species. However, disturbances may exert stress on lemurs and can affect their overall condition and immunocompetence. Some lemurs can only survive in undisturbed forests. Others prefer selectively-logged forests. But very few can live without forests. More people need to get involved in the protection of these primates and their habitats. Proper conservation actions, based on the results of this thesis and further investigations on the impact of natural or human-induced habitat alterations on primate populations,

can help to ensure the long-term viability of lemur species. Thereby, we can keep the raft called Madagascar, including its unique flora and fauna, afloat.

DUTCH

Lemuren op een zinkend vlot?

De ballast van antropogene verstoringen

Net als vele andere ecosystemen op aarde worden tropische bossen beïnvloed door een breed scala aan menselijke en natuurlijke factoren. Door een aanhoudend verlies van deze bossen zijn veel diersoorten kwetsbaar geworden en nemen de populatiedichtheden en verspreidingsgebieden van veel soorten af. De menselijke invloed op de leefomgeving is niet beperkt tot bossen op het vasteland, want ook de ontdekking van vele verafgelegen eilanden heeft geleid tot grote biotische en abiotische veranderingen ter plaatse. Door hun isolement herbergen veel eilanden een unieke en diverse inheemse flora en fauna en dit geldt zeker ook voor het eiland Madagaskar. Ondanks de nabijheid van het vasteland van Afrika kent dit eiland een andere biotische en geologische geschiedenis. Continentale drift heeft geleid tot de isolatie van Madagaskar en er hebben zich zeer uiteenlopende biomen ontwikkeld. Op drijvende vlotten van vegetatie, afkomstig van het vasteland, hebben de eerste gewervelde dieren zich miljoenen jaren geleden op het eiland gevestigd. Hier evolueerde de meerderheid van taxa geïsoleerd van verwante soorten, wat heeft geleid tot een hoog percentage endemische soorten, waaronder ook de lemuren. Lemuren behoren tot de onderorde van de halfapen (Strepsirrhini) en vertonen een aantal eigenschappen die als primitief worden beschouwd binnen de orde primaten ('Primates'). Ook de voorouder van deze lemuren wist ogenschijnlijk het kanaal van Mozambique over te steken en strandde op het enorme 'vlot' genaamd Madagaskar. Gedurende miljoenen jaren hebben lemuren verspreid over het eiland geleefd in uiteenlopende klimaten met grote verschillen tussen de seizoenen. Deze externe contrasten, in combinatie met een gebrek aan concurrentie van andere primaten en een beperkte predatiedruk, hebben bijgedragen aan de adaptieve radiatie van lemuren, waardoor er nu meer dan honderd verschillende soorten bekend zijn.

Historisch gezien vonden er uiteenlopende natuurverschijnselen plaats op Madagaskar, zoals extreme veranderingen in het klimaat en waterstanden, periodieke branden en vulkanische activiteiten. Tevens hebben mensen het landschap beïnvloed sinds ze zich ongeveer 2000 jaar geleden vestigden op Madagaskar, met ingrijpende gevolgen voor de inheemse soorten die hier leefden. Aangezien de overleving van lemuren momenteel onder druk staat door zowel menselijke als natuurlijke bedreigingen, is onderzoek naar de reactie van deze endemische halfapen op verschillende vormen van ecologische stress van belang. Om die reden streef ik er in dit proefschrift naar een overzicht te geven van de complexe relaties tussen de invloed van verschillende menselijke ingrepen en de aanwezigheid, het gedrag en de gezondheid van lemuren in gebieden verspreid over het eiland Madagaskar. Ik

onderzoek soorten die behoren tot het geslacht van de echte maki's, ook wel de bruine lemuren genoemd (geslacht *Eulemur*), aangezien deze soorten leven in uiteenlopende leefgebieden en in zowel ernstig verstoorde als intacte bossen. Naar mijn weten is deze geïntegreerde aanpak op verschillende geografische niveaus niet eerder toegepast in onderzoeken naar het effect van dergelijke verstoringen en biogeografische verschillen op relevante gezondheidsparameters van lemuren. De belangrijkste verwachtingen van dit proefschrift zijn (1) dat antropogene verstoringen in de vorm van selectieve houtkap invloed hebben op het aantal lemuren in een gebied en het naast elkaar bestaan van nauw verwante soorten kan faciliteren; en dat (2) deze verstoringen, evenals andere omgevingsfactoren, de bacteriële samenstelling en de aanwezigheid van nematoden in de darm van lemuren en hun afweersysteem kan beïnvloeden. Mijn proefschrift bestaat uit twee delen: allereerst onderzoek ik het effect van menselijke verstoringen op veranderingen in bosstructuren, de aanwezigheid van lemuren en het samen voorkomen van nauw verwante lemuursoorten. Daarna onderzoek ik de gezondheidseffecten van menselijke verstoringen en omgevingsfactoren, zoals het leven onder verschillende klimaatomstandigheden, op lemuren.

Hoofdstuk 1 vormt een algemene inleiding waarin ik mijn onderzoeksvragen uiteenzet en deze plaats in een theoretisch kader. Hier beschrijf ik tevens de biologische achtergrond van de lemuursoorten en de geografische gebieden waarin zij voorkomen. In **hoofdstuk 2** toon ik aan dat in bossen waar voorheen veel bomen zijn gekapt nog steeds duidelijk verschillen in bosstructuur en -samenstelling zichtbaar zijn en dat deze gekapte bossen nog niet volledig zijn hersteld ten aanzien van hun originele staat. Uit mijn resultaten blijkt echter dat zowel het aantal verschillende dagactieve lemuren als hun clustergroottes vergelijkbaar zijn in meer en minder intens verstoorde locaties in dit bos. De resultaten van dit hoofdstuk tonen dan ook aan, dat voorheen gekapte, herstellende bossen wel degelijk potentie hebben om te functioneren als habitat voor verschillende lemuursoorten en daarom belangrijk zijn in de bescherming van deze dieren. In **hoofdstuk 3** richt ik me op de ecologische verklaring van het samen voorkomen van twee nauwverwante lemuursoorten. Hierbij onderzoek ik de rol van het kappen van bossen, differentiatie in verschillende ecologische niches en directe competitie in het naast elkaar bestaan van deze soorten. De soorten behoren tot hetzelfde geslacht en vertonen veel gelijkenissen in zowel morfologie als in ecologische kenmerken. In dit hoofdstuk beschrijf ik de resultaten van een kwantitatieve gedragsstudie waarin ik de habitatkeuze van en directe competitie tussen deze soorten uiteenzet. De effecten van het kappen van bossen op bosstructuur en -samenstelling, zoals beschreven in hoofdstuk 2, kan heterogeniteit in bossen creëren. De twee nauwverwante lemuursoorten vertonen segregatie in bossen met verschillende gradaties van houtkap. Deze geografische

scheiding, in combinatie met nichedifferentiatie in termen van dieet, het moment van actief zijn en de voorkeurslocaties in bomen stelt deze nauwverwante soorten in staat om samen te kunnen voorkomen zonder elkaar weg te concurreren.

Vervolgens behandel ik in **hoofdstuk 4** de microbiële samenstelling in de darm van lemuren die in uiteenlopende gebieden van Madagaskar voorkomen. Hier laat ik zien dat biogeografische verschillen, en bijbehorende verschillen in dieet, grote invloed hebben op de samenstelling van de darmbacteriën van lemuren. De soort, leeftijd of het geslacht van een lemuur is hierbij van ondergeschikt belang. In **hoofdstuk 5** heb ik zowel het effect van omgevingsvariatie als van menselijke verstoringen onderzocht op de samenstelling van bacteriën en nematoden in de darm van verschillende lemuursoorten. Ik heb in alle onderzochte lemuurpopulaties nematodesoorten geïdentificeerd die behoren tot de geslachten *Callistoura* en *Lemuricola*. Tevens heb ik aangetoond dat nematoden van het geslacht *Lemuricola* meer aanwezig zijn in lemuren die leven in voorheen gekapte bossen. Biogeografische verschillen, seizoenen en de mate van boskap zijn factoren die de bacteriële samenstelling in de darm van lemuren beïnvloeden. Daarnaast heb ik aanwijzingen gevonden dat er een direct verband bestaat tussen de aanwezige nematoden en de samenstelling van bacteriën in de darm van lemuren. In **hoofdstuk 6** vergelijk ik de variatie in allelen (d.w.z. meerdere allelen per gen, waarbij een allel een sequentievariant is) binnen het deel van het genoom dat een belangrijke bijdrage levert aan de afweer van lemuren: het major histocompatibility complex II (MHC II). Uit ontlastings- en haarmonsters, die ik op een niet-invasieve manier heb verzameld van meerdere *Eulemur*-soorten in verschillende geografische gebieden, heb ik verlengde DNA-fragmenten in kaart kunnen brengen met een nieuw ontwikkelde DNA-primer. Hierbij heb ik vrijwel alle polymorfe codons, die coderen voor verschillende aminozuren, binnen de antigeenbindingsplaatsen geamplificeerd. Deze DNA-primer kan nu door andere onderzoekers worden gebruikt in immunogenetische onderzoeken van *Eulemur*-soorten en zeer waarschijnlijk ook van andere niet-menselijke primaten. Ik heb nieuwe MHC II *DRB*-allelen gedetecteerd met een hoge mate van zowel sequentie- als functioneel polymorfisme. Dit kan het resultaat zijn van een verschillende adaptieve selectiedruk op lemuurpopulaties die voorkomen in gescheiden geografische gebieden. Dit hoofdstuk ondersteunt tevens het verband tussen de aanwezigheid van parasieten en de diversiteit van het MHC II systeem. Daarnaast vond ik dat het aantal unieke allelen in de lemuurpopulatie die op een klein eiland leeft veel lager is dan in lemuren op het vasteland. Veel gebieden in Madagaskar worden gekapt en bossen gaan steeds meer lijken op eilanden, zowel qua oppervlakte als isolatie. Daarom is het monitoren van de MHC II-diversiteit van lemuren in deze 'eilanden van bos' een belangrijke indicator voor immuuncompetentie en men zou in het beschermen van lemuren het voorkomen van

een verlies aan genetische diversiteit moeten monitoren en proberen tegen te gaan.

In **hoofdstuk 7** breng ik de resultaten van dit proefschrift samen en bespreek ik andere uitdagingen waar de natuurbescherming in Madagaskar in de nabije toekomst voor komt te staan. De aanhoudende degradatie van het leefgebied van lemuren, in combinatie met een veranderend klimaat, bedreigt de overleving van lemuren en andere wilde dieren. Dit trekt de stabiliteit van het ooit stabiele 'vlot' Madagaskar in twijfel. Om de lemuren van Madagaskar te beschermen voor de vele bedreigingen is succesvol natuurbeheer nog nooit zo belangrijk geweest.

In dit proefschrift heb ik gevonden dat, ondanks dat het kappen van bossen zowel de bosstructuur als -samenstelling langdurig verandert, deze herstellende bossen vanuit een functioneel perspectief waardevol zijn, namelijk als habitat voor lemuren. Dergelijke verstoringen kunnen ook verschillen in landschap creëren, die het samenleven van nauwverwante lemuursoorten mogelijk maakt. De verstoringen kunnen echter ook stress opleveren voor de lemuren en daarmee de algehele conditie en weerstand tegen ziekten benadelen. Antropogene verstoringen en biogeografische verschillen beïnvloeden de samenstelling van bacteriën in de darm, de aanwezigheid van darmnematoden en de MHC II-diversiteit van lemuren, wat hun overlevingspotentieel kan verminderen. Het monitoren van aanwezigheids-, gedrags- en gezondheidsparameters van lemuren is daarom belangrijk om vroegtijdig signalen van stress of een verminderde gezondheid op te merken. Er zijn lemuursoorten die alleen kunnen overleven in niet-verstoorde bossen. Andere lemuren geven juist de voorkeur aan enigszins verstoorde bossen. Maar één ding is zeker, vrijwel geen enkele soort kan overleven zonder bos. Om deze bijzondere halfapen en hun natuurlijke leefomgeving te beschermen moeten zoveel mogelijk mensen bewust worden van het belang van natuurbescherming. Succesvolle beschermingsmaatregelen, gebaseerd op de resultaten van dit proefschrift en toekomstige onderzoeken naar de menselijke invloed op primatenpopulaties, kunnen bijdragen aan het garanderen van de overleving van soorten op lange termijn. Op deze manier proberen wij samen alles te doen om het vlot genaamd Madagaskar, met haar unieke flora en fauna, drijvend houden.

MALAGASY

Ho rendrika ve ny varika/gidro?

Ireo karazana fikorontanana nataon'ny olombelona

Toy ireo rindran-drafi-boary an-tanety, ny ala tropikaly dia ianjadian'ny vokatr'ireo loza Voajanahary sy ny asan'olombelona. Noho ireo fitohizan'ny faharavan'ireo ala ireo, dia maro ireo karazan-javaboary tandidomin-doza ny fahavelomany, ary ny hamaroany sy ireo toerana ahitana azy dia miha mihena hatrany foana. Ny fampiasana miendrika fanimbana ataon'ny Olombelona amin'ny tontolo iainana dia tsy voafetra teo amin'ny alan'ire tanibe ihany. Ny fahitana ireo nosy an-jatony maro miparitaka manerana ny ranomasim-be eran-tany dia nitarika fiovana ny endrika natioraly sy ny zava-boary zanatany nisy teo. Noho ny fitokanan'ireo nosy ireo dia nanjary lasa toerana nitahiry ny rohin-drafi-boary ny nosy ho an'ireo zava-manan'aina biby sy zava-maniry zanatany. Na dia tsy lavitra ny kontinanta afrikana aza i Madagasikara dia nanana ny tantarany manokana mombo ny olombelona sy ny zavaboary izay tena nampiavaka azy. Ny fikisahany tamin'ireo tanibe dia nahatonga azy tafasaraka tamin'ireo ka lasa nosy, ary nanomboka teo no nivoaran'ireo zava-manan'aina nisy tao aminy. Ny fiangonan'ireo biby manana hazon-damosina teto Madagasikara dia inoana fa avy tamin'ireo zavamaniry nitsingevana teny ambonin'ny ranomasim-be izay niseho efa an-tapitrisa taona lasa. Ny ankamaroan'ireo karazan-javaboary dia nivoatra manokna mihitsy ka mety ho tsy nitovy tamin'ireo fototra nihaviany, ka nahatonga ireo endrika hafa nahazanatany azy ireo. Teo amin'izany fitsingevanan'ireo zavamaniry teny ambony ranomasina izany no nahatongavan'y razamben'ireo varika teto amin'ity nosy antsoina hoe "Madagasikara" ity. Ny varika dia ao anatin'ny sokajin'ny Strepsirrhini izay manana toetra izay heverina fa nisy teo amin'ireo nipoiran'ny vondrona Primates. Koa nandritry ny aona maro an-tapitrisany dia nisedra fiovaovana be momba ny toetr'andro sy ny fivoaran'ny tany nanerana ny nosy ny varika, ka nahatonga ny fisian'ireo karazany maro misy ankehitriny.

Ara-tantara, ny fiovan'ny tontolo iainana teto Madagasikara dia vokatry ny fiovana tampoka nisy, ireo fiovan'ny toetr'andro tena mifanalavitra, ireo afo miverina matetika, fihetsehan'ny ranomasina, ny fipotrahan'ny volcano ary ny fiovna hita teo amin'ny sisin'ny moron-tsiraka. Fiovana lehibe no nitranga ka nanova ny endriky ny tany sy niteraka endrika tontola hafa mihitsy rehefa tonga ny Olombelona 2000 taona lasa. Izay fiovana izay dia nisy fiantraikany lehibe teo amin'ireo zavaboary zanatany. Ny fahaveloman'ireo varika ankehitriny dia misedra olana goavana vokatr'ireo fahapotehina nataon'ny olombelona, izay niampy ireo loza ara-boajanahary. Vokatr'izay dia ilaina ny manao fikarohana eo amin'ny fomba hamalian'ireo zavaboary zanatany ireo ny endrika samihafa misy eo amin'ny tontolo misy azy. Noho izany, ny tanjon'ity asa fikarohana (these) ity dia nafahana nampiseho niaraka ny fifandraisana sy

rohy maro maro sy mifandray amin'ny endrika manahirana teo amin'ny seho vokatry ny asan'ny olombelona sy ny ara-boajanahary teo amin'ny lafiny ray, sy ny fisian'izany, ary koa ny toetra famaliana ny tranga miseho, sy ny fahasalaman'ny varika teo an-daniny, izay asa natao nanerana an'i Madagasikara. Araka ny fahafantarako dia ity fikarohana ity no anisan'ny voalohany nijery izay fiasa nampiatra fomba fijery miaraka ka nampiasa ny vokatry ny fahasimbana nataon'ny olona sy ny fanamby ara-tontoloianana ka nampiditra ny fisian'ireo singa momba ny fahasalaman'ireo karazam-barika *Eulemur* ireo, ka nojerena tao anatin'izany ireo ambaratonga ara-jeografika. Ny petra-kevitra nojerena tao anatin'ity fikarohana ity dia: (1) Ny fanimbana ataon'ny olona (oh. Fakàna ireo hazo isan-karazany fantenan'ny olona); (2) Ireo fikorontanana vokatry ny fanimbana miampy ny tranga ara-boajanahary dia miteraka koa fiovana amin'ny fomba fitambaran'ny mikraoba, ny toetry ny parasitan'ny tsinay, sy ny karazana rohy lehibe sy manahirana eo amin'ny fafandraisan'ireo (CHM) sokajy (*DRB*) eo amin'ny varika.

Fizarana roa no hita ato amin'ity asa fikarohana ity: Voalohny dia ny fampisehoana ny fifandraisan'ny vokatry ny fanimbana nataon'ny olona miaraka amin'ny fiovan'ny rafitry ny ala, ny fahabetsahana/fahavitsihin'ny fahitana ny varika, ary ny fiarahan'ireo karazany samihafa. Faharoa dia ny fomba amalian'ny varika manoloana izay fahasimban'ny tontolo misy azy izay sy ireo fanamby samihafa misy eo amin'ny tontoloiainana. Ny **toko 1** dia fampidirana amin'ny ankapobeny ka mametraka ny fototry ny asa amin'ny fampisehoana ireo famaritana teorikan'ity asa ity sy ny fahalalana biolojika ankehitriny momba ny karazam-barika nanaovana fikarohana ary ny toerana nanaovana ny fikarohana. Ny **toko 2** dia nijerena ny vokatry ny fanimbana nataon'ny olombelona teo amin'ny rafitry ny ala sy ny karazan-kazo mandrafitra izany, ny fahabetsahana/fahavitsian'ny fahitana ny biby ary ny habetsahan'ny vondrona/fianakaviana miaraka ho an'ireo izay mandeha amin'ny atoandro eo anivon'ny toerana iray. Eny fa na ho an'ireo ala notrandrahana ka tsy nahitana ireo endrika nisy tany am-piandohana aza, dia nahitana kosa fa mbola maniry ka mety mbola ho lasa toerana tsara nafahan'ny varika mbola mivelona. Noho izany dia ny habetsahan'ny fahitana sy ny haben'ny vondrona miaraka dia mitovy teo amin'ireo ala nisy fahasimbana sy ny ala tsy dia simba loatra. Ny valin'ity asa fikarohana ity dia nahitana fa tsy misy fahasamihafana teo amin'ny ala notrandrahana efa taloha efa ela sy ireo izay andalam-paniriana indray ka samy mbola afaka mandray tsara ireo karazam-barika afaka miaraka mivolona amin'ilay ala. Ny **toko 3** dia maneho ny anjara toerana eo amin'ny fahasimbana, ny fahasamihafan'ny ala, sy ny fifaninanana mivantana teo amin'ny fiarahana eo amin'ny karazam-barika roa samihafa nefa mitovy fototra ary miara-monina, ka mizara toetra iraisana ara-ekolojika maro. Ity toko ity dia mirakitra ny vokatry ny fikarohana toetran'ny biby eo amin'ny lafiny fifidianana ny toerana ampiasaina sy ny fifaninanana mivantana eo amin'ny karazany samihafa. Raha ampitahaina amin'ny toko 2 dia misy ireo karazana endrim-pahasimbana izay miteraka

fahasamihafana eo amin'ny fampiasana ny toerana ampiasaina ao anaty ala. Karazana varika roa samihafa nefa iray fototra dia nahitana tsy fitovizana lehibe ny toerana ampiasaina ao amin'ny karazam-paritra maro izay lasa tontolo tena niova be tokoa vokatry ny fitrandrahna ny ala. Izay "fisarahana" miaraka amin'ny fahasamihafana amin'ny fampiasana ny toera-ponenana amin'ny fotoana samihafa ny sakafo izay koa eo amin'ny karazam-barika, sy ny fifandraisana miendrika fifandroahana dia manamora ny fiarahan'ireo varika samihafa iray fototra.

Ny **toko 4** dia natokana iresahana ny fiombonn'ny mikraoba hita amin'ny tain'ny varika samihafa hita tamin'ny toerana samihafa. Ny vokatry ny fikarohana ia nampiseho fa ny fahasamihafan'ny toerana sy ny sakafo hita amin'ilay toerana no antony lehibe indrindra mamaritra ny fiombonan'ny mikraoba hita koa amin'izany toerana izany ho an'ny varika, fa tsy dia noho ilay karazam-barika loatra na ny maha-lahy na vavy azy. Ny **toko 5** dia mampivoitra ny fanadihadihana ny vokatry ny fiovan'ny tontolo sy ny fahapotehana nataon'ny olona teo mikraoba bakiteria hita amin'ny tain'ny biby sy ny parasitan'ny tsinain'ny varika manerana an'i Madagsikara. Ny fidiran'ny "némátodes" karazany *Callistoura* sy *Lemuricola* dia saika hita teo amin'ny vondrona varika rehetra, fa ny fitoeran'ny *Lemuricola* dia tena maro dia maro tamin'ny ala efa notrandrahana. Ny fahasamihafan'ny toerana, ny fotoana, ary ny fitrandrahan'ala dia niantoka koa ny fahasamihafan'ny fiombonan'ny mikraoba bakteria miaraka. Ity fikarohana ity koa dia nanasongadina ny fifandraisan'ny parasitan'ny tsinay sy ny fitambaran'ny mikraoba bacteria. Ny **toko 6** dia maneho fampitahana ny fahasamihafana manahirana ny "allèles" eo amin'ny ireo afaka miara-miaina (CHM) sokajy II eo amin'ireo karazana *Eulemur* hita eo amin'ny toerana samihafa. Ny fakana ampahany amin'ny tay sy ny volo dia nafahana nijery silaka ADN lava tamin'ny alalan'ny teknika vaovao noforonina. Izany teknika nampitombo ny "acides aminés polymorphes" mitazona "antigènes" izany dia azo ampiasaina amin'ny fikarohana any aoriana momba ny fahasalamana ara-jenetika (CMH II *DRB*) ny karazana *Eulemur* sy mety koa ireo karazana primates hafa ankoatry ny olona. Ity fikarohana ity dia nahitana "allèles" vaovao MHC II DBR izay mety avy tamin'ny karazana tsindry voatokana nipoitra avy tamin'ny aretina ho an'ireo vondrona varika mivelona amin'ny toerana samihafa.

Misy singa tena mazava tsara maneho ny fifandraisana eo amin'ny parasita sy ny karazana CHM sokajy II koa nivoitra tamin'ity fikarohana ity. Hita ihany koa fa ny isan' ireo "allèles" mitokana hita ao amin'ny vondrona varika amin'ny nosy kely dia vitsy noho ireo any amin'ny tanibe. Tsara ny mahalala fa ny potik'ala ia mitovy ihany toy ny nosy eo amin'ny lafiny habe sy fitokanana, fiambenana ny karazana CHM sokajy II izay hamantarana ny fisian'ny fifaninanana eo amin'ny singa miaro. Ny paika fiarovana mampihena ny fahaverezana genetika eo amin'ny varika dia tokony ho atsanganna. Ny **toko 7** dia fampiarahana ny vokatr'ity fikarohana ity sy fametrahana

fanamby hafa ho amin'ny fiarovana ny zavaboary eto Madagasikara. Ny fihenana sy fahapotehan'ny tontolo fonenan'ny varika izay tsy misaraka amin'ny foovan'ny toetr'andro dia mipetraka ho loza mitatao ho an'ny varika sy ireo karazam-biby hafa. Ary mbola mampametram-panontaniana ny fisian'ny filaminana eto Madagasikara daholo izany. Koa afahana miady amin'ireo zavatra maro tsy maintsy sedrain'ireo zavaboary maro eto Madagasikara ireo dia tokony hisy fitantanana tsara ho ajoro ho an'ny tontoloiainana.

Ity asa fikarohana ity dia tena mampiseho fa na dia ny fikorontanana vokatry ny asan'ny olombelona aza dia mety hitarika fiovana ny endrika sy ny karazan-kazo mandrafitra ny ala sy ny fahafahany maniry sy mitombo ka mety hanana antoka lehibe eo amin'ny lafiny fiarovana noho ny ala toeram-ponenan'ny varika. Ireo karazam-panimbana ireo koa dia mety hiteraka fahasamihafana lehibe ny tena endriky ny ala tany am-piandohana ka manamora ny fiarahan'ireo varika samihafa nfa iray fototra. Ny fahasimbana koa dia mety hiteraka vesatra/fahosana amin'ny lafiny maro eo amin'ireo varika ka hisy fiantraikany amin'ny toe-batany sy fahasalamany ary koa ny fahafahany miatrika sy miady amin'ny aretina. Ny fahapotehana ataon'ny olona sy ny fahasamihafan'ny toerana dia misy fiantraikany amin'ny fiombonan'ny mikraoba eo amin'ny varika, ny fetran'ny parasite eo amin'ny tsinay, ary ny karazana CHM sokajy II. Izany fiovana izany dia mety hanalefaka ny fahafahan'ny varika miaitra sy miady min'ny tsindry hafa mety hisy, ary vokatr'izay dia mety hisy fiantraikany mihitsy amin'ny fahavelomany izany. Ny fijerena ny fisiany, ny toetrany, sy ireo endrika hafa afahana manombana ny fahasalaman'ny varika dia tena zava-dehibe tokoa hijerena koa ny rajako amin'ireo toetra mampiseho tranga mampanahy na manalefaka vokatr'izay olana atrehin'ilay biby izay. Misy ireo karazam-barika izay ty afaka mivelona raha tsy amin'ny ala izay tsy nisy fahasimbana ihany. Vitsy ihany ireo izay afaka miaina ivelan'ny ala. Tokony hisy ny olona izay miasa mitohy ho fiarovana ireo primates ireo sy ny toeram-ponenany. Mila asa firovana tena mifanaraka amin'ny zava-misy ka tena mamaha ny olana izay avy amin'ny vokatr'ity asa fikarohana ity sy ireo hafa izay nijery ny vokatry ny fahapotehan'ny ala Voajanahary na vokatry ny asan'ny Olombelona amin'ny primates, ka afahana miantoka ny fahavelomana maharitra ireo izay atahorana ho lany tamingana. Ary noho izany dia ho afaka hitahiry ireo zavaboary biby sy zavamaniry isika sy hitomboan'izy ireo.

FRENCH

Le naufrage des lémuriens?

Le ballast des perturbations anthropiques

Comme tous les écosystèmes naturels terrestres, les forêts tropicales sont affectées par de nombreux effets naturels et anthropiques. En raison de la perte continue de ces forêts, beaucoup d'espèces sont devenues vulnérables, leurs densités de populations et leurs répartitions sont en déclin. Les interférences des Hommes avec l'environnement ne sont pas limitées aux forêts continentales. La découverte de centaines d'îlots isolés éparpillés dans les océans mondiaux a conduit à la modification des paysages et à des changements biotiques. Du fait de leur isolement, les îles sont souvent le berceau d'un éventail d'écosystèmes accueillant des espèces fauniques et floristiques endémiques. Malgré sa proximité avec le continent africain, l'île de Madagascar possède une histoire humaine et biotique très différente. La dérive des continents a mené à son isolation, et depuis des biomes distincts se sont développés. La colonisation de Madagascar par les vertébrés a probablement eu lieu via la dispersion d'îlots de végétation flottants à travers les océans il y a des millions d'années. La majorité des taxons a évolué indépendamment de leurs plus proches parents, menant à un fort endémisme des espèces. En flottant sur de tels îlots de végétation, un ancêtre des lémuriens s'est échoué sur un énorme « îlot », appelé Madagascar. Les lémuriens, un clade des primates Strepsirrhini, possèdent des caractéristiques considérées ancestrales pour l'ordre des Primates. Durant des millions d'années, les lémuriens ont dû faire face à d'extrêmes différences de climat et de géologie à travers l'île et à travers les saisons, ce qui a contribué à leur rayonnement en plus d'une centaine espèces différentes.

Historiquement, les variations environnementales naturelles à Madagascar incluent des changements climatiques abrupts, des évènements météorologiques extrêmes, des feux périodiques, des dynamiques océaniques, des éruptions volcaniques et des modifications de la ligne littorale. Des modifications anthropiques du paysage et des changements écologiques majeurs sont apparus à l'arrivée de l'Homme sur l'île de Madagascar il y a 2000 ans. Ces changements ont eu des conséquences considérables pour les espèces indigènes. La survie des lémuriens est aujourd'hui menacée par une pression anthropique intense, qui s'ajoute à des effets naturels. Par conséquent, il est urgent d'étudier les réponses de ces espèces endémiques à différentes formes de stress écologique. Ainsi, cette thèse a pour objectif de donner une vue d'ensemble des relations complexes entre les multiples effets anthropiques et naturels d'une part, et de la présence, du comportement, et de la santé des lémuriens d'autre part, ceci à travers l'île de Madagascar. À ma connaissance, cette étude sera la première à adopter une perspective intégrée,

mettant en relation les effets des perturbations anthropiques et les challenges environnementaux avec la présence et les paramètres de santé des espèces de lémuriens du genre *Eulemur*, à différentes échelles géographiques. Les principales prévisions de cette thèse sont: (1) les perturbations anthropiques (ex. l'exploitation forestière sélective) altèrent les taux de rencontre des lémuriens et facilitent la coexistence d'espèces apparentées; (2) ces perturbations ainsi que les challenges naturels influencent la composition du microbiome, le niveau de parasitisme gastro-intestinal, et la diversité des complexes majeurs d'histocompatibilité (CMH) de classe II *DRB* chez les lémuriens.

Cette thèse est composée de deux parties : premièrement, la mise en relation des impacts des perturbations anthropiques avec les changements structuraux des forêts, les taux de rencontre des lémuriens, et leur coexistence. Deuxièmement, les réponses physiologiques des lémuriens face aux perturbations anthropiques et aux challenges environnementaux. Cette thèse est composée de deux parties : premièrement, la mise en relation des impacts des perturbations anthropiques avec les changements structuraux des forêts, les taux de rencontre des lémuriens, et leur coexistence. Deuxièmement, les réponses physiologiques des lémuriens face aux perturbations anthropiques et aux challenges environnementaux. Le **chapitre 1** est une introduction générale posant le contexte à travers une présentation du cadre théorique de cette étude et des connaissances biologiques actuelles sur les espèces et les zones géographiques étudiées. Le **chapitre 2** est consacré aux impacts des perturbations anthropiques sur les structures forestières et sur la composition, les taux de rencontre et la taille des groupes de toutes les espèces diurnes au sein d'une communauté de lémuriens. Même si les forêts exploitées pour l'industrie du bois n'ont pas retrouvé leurs conditions originelles, elles semblent s'être régénérées sur le plan fonctionnel, de telle sorte qu'elles sont devenues un habitat favorable pour les lémuriens. En effet, les taux de rencontre des lémuriens et la taille de leurs groupes sont similaires entre les sites perturbés et moins perturbés. Les résultats de cette étude suggèrent que les forêts anciennement exploitées et en cours de régénération ont un potentiel considérable d'accueil des communautés de lémuriens. Le **chapitre 3** traite du rôle des perturbations anthropiques, de la différenciation de niche, et de la compétition directe dans la coexistence stable de deux espèces de lémuriens congénériques et sympatriques, partageant de nombreux traits écologiques. Ce chapitre contient les résultats d'une étude comportementale quantitative sur la sélection de l'habitat et la compétition directe entre les espèces. En référence aux résultats du chapitre 2, divers évènements perturbateurs peuvent créer une hétérogénéité spatiale dans les forêts. Deux espèces de lémuriens congénériques montrent une ségrégation spatiale à grande échelle dans différentes zones d'un environnement rendu hétérogène par l'exploitation forestière. Cette ségrégation,

associée à la différentiation de l'utilisation spatiale et temporelle de la ressource entre les espèces, ainsi qu'aux interactions agonistiques, facilite la coexistence des espèces de lémuriens congénériques.

Le **chapitre 4** est consacré à la composition microbienne des fèces de différentes espèces de lémuriens présents dans différents habitats. Les résultats de cette étude montrent que les différences biogéographiques, et les régimes alimentaires associés, sont les principaux facteurs influençant la composition du microbiome des lémuriens, plutôt que l'espèce hôte ou le sexe. Le **chapitre 5** présente une analyse des effets des variations environnementales et perturbations anthropiques sur le microbiome bactérien fécal et les parasites gastro-intestinaux des espèces de lémuriens à travers Madagascar. Les infections par les nématodes des genres *Callistoura* et *Lemuricola* ont été trouvées dans toutes les populations de lémuriens, et les infections dues au genre *Lemuricola* étaient particulièrement fortes dans les forêts ayant été exploitées. Les variations biogéographiques, la saisonnalité, et l'exploitation des forêts sont les déterminants de la composition du microbiome bactérien. Cette étude a également mis en évidence un effet interactif entre les parasites gastro-intestinaux et la composition du microbiome bactérien. Le **chapitre 6** présente une étude comparative sur la variation allélique des complexes majeurs d'histocompatibilité (CMH) de classe II entre les multiples espèces du genre *Eulemur* dans différentes zones géographiques. La collecte non-invasive d'échantillons de fèces et de poils a permis de génotyper des fragments d'ADN allongés à l'aide d'un set d'amorce nouvellement développé, amplifiant quasiment tous les acides aminés polymorphes des sites de fixation d'antigènes. Ce set d'amorce peut être utilisé dans de futurs examens médicaux sur l'immunité génétique (CMH II *DRB*) des espèces du genre *Eulemur* et probablement pour d'autres espèces de primates non-humains. Cette étude a permis de détecter de nouveaux allèles MHC II *DRB* avec un haut niveau de séquence et de polymorphisme fonctionnel, ce qui pourrait provenir de diverses pressions de sélection adaptative, générées par des pathogènes, sur les populations de lémuriens vivant dans différentes zones géographiques.

Des éléments tangibles supportant l'existence d'un lien entre la présence de parasites et la diversité des CMH de classe II ont également été mis en évidence. De plus, le nombre d'allèles singuliers présents dans les populations de lémuriens vivant sur une petite île était bien plus bas que dans les populations vivant sur le continent. Sachant que les fragments forestiers sont similaires aux îles en termes de surface et d'isolement, la surveillance de la diversité des CMH de classe II est un indicateur important de l'immunocompétence. Des plans de conservation atténuant la perte de diversité génétique chez les lémuriens doivent par conséquent être établis. Le **chapitre 7** est une synthèse des résultats de cette thèse et discute d'autres challenges que la conservation de la biodiversité qu'aura à affronter Madagascar dans

un futur proche. Le déclin et la dégradation de l'habitat des lémuriens, associés au changement climatique, menacent le futur des lémuriens et d'autres espèces fauniques. Ceci remet en question la stabilité de Madagascar. Afin de surmonter les nombreuses menaces affrontées par les espèces, une bonne gestion de la nature n'a jamais été aussi importante.

Cette thèse démontre que même si les perturbations anthropiques peuvent altérer la structure et la composition des forêts, leur régénération peut avoir un potentiel de conservation considérable en tant qu'habitat pour les lémuriens. De telles perturbations peuvent également créer une hétérogénéité de paysages qui facilite la coexistence d'espèces de lémuriens congénériques. Les perturbations peuvent générer du stress chez les lémuriens et ainsi affecter leur condition générale et leur immunocompétence. Les perturbations anthropiques et les différences biogéographiques ont un impact sur le microbiome des lémuriens, le niveau de parasitisme gastro-intestinal, et la diversité des CMH de classe II. De tels changements peuvent altérer la capacité des lémuriens à supporter des pressions additionnelles, et par conséquent leur capacité de survie. Le contrôle de la présence, du comportement, et des paramètres de santé des lémuriens est par conséquent important pour détecter en amont des signes de stress alarmants ou un affaiblissement. Certains lémuriens ne peuvent survivre que dans des forêts n'ayant pas subi de perturbations, tandis que d'autre préfèrent les forêts ayant subi des coupes sélectives. Mais très peu peuvent vivre en dehors des forêts. Davantage de personnes doivent être impliquées dans la protection de ces primates et de leurs habitats. Des actions de conservation appropriées, basées sur les résultats de cette thèse et sur d'autres études traitant de l'impact des altérations d'habitat naturelles ou issues de l'Homme sur les populations de primates, peuvent aider à assurer la viabilité à long terme des espèces menacées. Ainsi, nous pourrons maintenir la faune et la flore uniques de Madagascar à flot.

Thank You

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MADAGASCAR

STUDENTS

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Other MSc students that spent hours in the lab for their thesis and that I really want to acknowledge are Jolien, Nikki, Maartje, and Tamar. You all did very thorough and elaborate studies on genetics, microbiota, or parasites. I want to show some very special thanks to Tamar. Tamar, you did an incredible job with your genetic MSc thesis at the BPRC. I was really impressed by your dedication and I really appreciated collaborating with you and writing a paper together. I wish you all the best at the University of Ulm and I would be happy to collaborate in future projects in conservation genetics as well. Then, Coco, Sherdina, Marlies, and Anne, thanks for your motivation, interest, and enthusiasm while doing your BSc thesis within my project.

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I truly could not have done any of this work without the help and friendship of the amazing research technicians, staff, field assistants, and students I had the privilege of working with at Centre ValBio (CVB). For their advice and support in the field, I want to thank Patricia Wright, Eileen Larney, and John Cadle. I would especially like to thank CVB director Eileen for her tireless work maintaining a magnificent research environment and for her inspiring ideas. I will never forget the frozen chicken and peanut butter cake during one of our lonely Christmas nights in the field. John, thanks for handling our Dutch 'directness', for all the rat traps we needed, and your very much appreciated smile when we found you some dead reptiles.

Many thanks to the CVB staff, and especially to Nemese Randriarimanana,

Mamisoa Ramarjaona, Heritiana Louisette, Richard Ramarjaona, Pascal Rabeson, Prisca Oliva Andriambinintsoa, Jeanne Julia Rasoarimamonjy, Jean Claude Razafimahaimodison, Florent Ravoavy, Jean de Dieu Ramanantsoa, and Désiré (Dede) Randrianarisata. I want you to know that I am very grateful for introducing me to a community of incredibly brilliant and inspiring scientists, for the fantastic atmosphere, for the great friends I met, and for making me feel right at home in Madagascar. Thanks for helping out in so many aspects of the fieldwork, including preparing camp expeditions (e.g., in arranging porters, transport, and food) and in all other logistical and administrative tasks. Also, thanks for your kindness and for all the jokes about rain, leeches, and the always travelling, disappearing, or hiding lemurs. And, of course, I would like to thank the CVB musicians for the nice music and the kitchen staff for preparing the good meals during the nights we 'recovered' from camp expeditions at the research station: Solo Ramorasata, Madame Angele Ravaonirina, Mariette Ravaonirina, Bery, and Lemanana. I still remember this sequence, going from 'Noana aho' to 'Hmm, tsara!' to 'Voky be!' to 'Thank you Mariette!' to 'Tsara manji!'. Thank you for all the evenings when we ate, danced, and laughed.

FORMAL

The field work in Madagascar would not have been possible without the kind permission and cooperation of the Department of Paleontology and Biological Anthropology at the University of Antananarivo, with special thanks to Prof. Andriamialison Haingoson. I further like to thank the Institute for the Conservation of Tropical Environments (ICTE) and the Madagascar Institute pour la Conservation des Ecosystèmes Tropicaux (MICET), and especially Benjamin and Tina, who were very helpful during the entire process from applying for permits to flying home with research samples. Thanks go to the Malagasy government and Madagascar National Parks (MNP), the Commission Tripartite and the CAFF of the Direction des Eaux et Forêts, Centre de Formation Professionelle Forestière (CFPF), the Mitsinjo Association, and CNFEREF Morondava for their formal permission. I would also like to thank Peter Kappeler from the departement of Behavioral Ecology and Sociobiology at Deutsches Primatenzentrum (DPZ), who kindly allowed me to do fieldwork based in his research station in Kirindy and Léon for his help with logistics, including some problems with Air Madagascar.

GUIDES

I want to thank all field technicians and guides that helped me collecting data in various national parks, special reserves, and other forest areas. Collecting thousands of faecals and finding lemurs, trails, or our camp, would not have been possible without the invaluable help of many people, including the guides from CVB: Telo

Albert, Razafitsiafajato Armand, Razafindraibe Faustin Jean Guy, Randrianantenaina Donné Lucien, Rasendrinirina Victor, Rakotonirina A. Thierry Emile (Nirina), Rakotoniaina Jean Félix (Rakoto), Solo Justin, Randrianirina Georges René, Rakotovao Rémi, Tombotiana Aimé Victor, and Lahitsara Jean Pierre. Also, a big thanks to the MNP guides, including Emile, Laurent, and Diamondra, for the team-work of spotting lemurs in Talatakely and Theo for joining me on our expedition to find the aye-aye. Then, I would like to thank Tina (Kirindy), Tianasoa Honoré Ratolojanahary (Mitsinjo), George (Andasibe), Tina Ratovonjanahary and Paul (Ankarafantsika), Nicolas Salo Dire (Ankarana), Tiana Andriantsihoarana (Andringitra), Toussaint (Isalo), Edmond (Nosy Kkomba), the guides from Zombitse and Montagne d'Ambre, our handsome guides with their pirogues from Nosy Be, and all other guides that I worked with. Thanks for all your assistance in fieldwork, indispensable knowledge, expertise, friendship, and the cheerful atmosphere you created in the field or in camp. Also, thanks to the cooks in camp, like Jocelyn and Lova, who made the best rice and beans with Ranon'apango (i.e., burned-rice water) ever. I am sure I will see you all soon again!

I also wish to thank many other people that I was lucky enough to live with in the forest for extended periods of time, including: Jonno, Tara, Martina, Marloes, Hanitra, Falke, Tim, Ricardo, René, Samuel, Sam, Silvija, Jesse, Aaron, and many others. Thanks for having the privilege of enjoying your companionship! And of course: all of the Study Abroad and Respect students, thank you for your enthusiasm, dances, and songs. James Herrera and Lydia Tongasoa, thanks for welcoming us in camp Vohiparara and I will never forget our expedition to Miaranony together. I would further like to thank the many drivers that drove us throughout the island, slipping down hills, getting stuck on mountains, and changing so many tyres. Thank you, Thom, Zina, Fano, Tina, and Paul.

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Svalbard. Dimby Raharinjanahary and Jonah Ratsimbazafy, thanks for your friendship and support in Madagascar. Then, Doxy, misaotra betsaka for hosting me at Nosy Komba and Sonja for your nice songs and the nights dancing on the beach. Baie dankie, Marcine Cooper for your delicious meals, like rice in coconut milk with fresh vanilla, that you cooked for us when we came back from the slippery forest and your interest in the lemurs living in your little paradise. Thanks Saka Manga, for making me feel home in the hectic city of Antananarivo. TBA, and especially Nigel Mann, Anthony Kuria, and Clive Nuttman, for sparking my interest in nature conservation. Thanks also to the other *Eulemur* specialists that I had the honour to meet in Hanoi or Madagascar. Thanks for sharing experiences with and fascination for the little brown lemurs: Benjamin Freed, Steig Johnson, Guiseppe Donati, Rachel Jacobs, and Stacey Tecot.

The generous support of several granting agencies also made this research possible. Therefore, I want to thank all board members of FONA Conservation, and especially Dr. Henk Smit, Dr. Loek Kuiters, and Ir. Guus Schutjes, for their advice and enthusiasm and to offer me and many of my MSc students the opportunity to do their MSc projects in Madagascar.

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SHORT BIOGRAPHY

Ever since Iris de Winter was a child, she has been interested in the natural world and was fascinated in all natural forces that drive the processes we see everywhere around us. Because of this passion for nature, she started her study Biology at the Wageningen University in the Netherlands in 2007 and specialised in (marine) ecology and nature conservation. After an international conservation course of the Tropical Biology Association in Madagascar, she became intrigued by the diversity in ecosystems and the unique endemic flora and fauna present. Why are there so many different species of primates? This broad question led to her first MSc thesis project on the competition between closely-related lemur species. For her second MSc thesis, she performed half a year of dive research, as she was also very interested in understanding, exploring, and preserving the underwater world. She studied the behaviour, activity patterns, and substrate use of green turtles in a marine protected area off the coast of East Kalimantan, Indonesia. While living on the small and isolated island Derawan, she became increasingly aware of the effects of irresponsible marine activities. She became concerned about the natural world and the sustainability of all the living resources and developed an even greater motivation to preserve threatened ecosystems.

After finishing her BSc and MSc (both *cum laude*), she was still very much interested in the big contrasts on the island Madagascar. On the one hand, the anthropogenic damage to the natural environment was striking. On the other hand, the special geological history, endemic wildlife, and the extraordinary natural diversity is unparalleled. She received a competitive, personal research grant from Production Ecology and Resource Conservation. This enabled her to start her doctorate study on the impacts of anthropogenic habitat changes on the health of lemurs. In this PhD project, she gained substantial experience with data collection, analyses, and management and published different scientific articles, obtained multiple research grants, and presented her work at national and international conferences. She also enjoyed supervising 30 MSc and 4 BSc students in their thesis and liked coordinating multicultural and interdisciplinary research teams while working in different isolated

and primitive field sites throughout Madagascar.

During her study and PhD, she organised and coordinated student conservation courses in Southern Africa for both undergraduate and graduate students and worked as student coach, workshop leader, and as a student assistant in many different courses. During the first phase of her PhD, she had been chair of the student staff council (SSC/GV) and vice-chair of the WUR Council for one year. In these councils, she represented the interests, well-being, and position of students and employees in developments and policy within Wageningen University. Here, she learned about the functioning and decision-making process of a large organisation such as a university. During the second part of her PhD, Iris worked as incubation manager at StartLife for a year. She was responsible for overseeing the incubation program and became competent in supporting innovative start-ups in the sector food & agri.

Iris currently works as a coordinator of the course 'Wildlife Conservation' at Utrecht University; as research coordinator for Apenheul Primate Park in a conservation project on blue-eyed black lemurs; and as a tour manager, ecotourist guide, and primate-expert for several travel agencies that explore the fascinating natural world. In the future, she aims to work in science-based conservation and in this way to contribute to the preservation of threatened natural areas and the vulnerable species that rely on them.

SELECTED PUBLICATIONS

de Winter, I.I., van der Hoek, S., Schütt, Heitkönig, I.M.A., van Hooft, W.F., Gort, G., Prins, H.H.T., Sterck, F. (2018) Anthropogenic disturbance effects remain visible in forest structure, but not in lemur abundances (*Biological Conservation* 2018, accepted and in press, manuscript ID: BIOC_2017_784)

de Winter, I.I. and Umanets, A., IJdema, F, Garcia, J.R., van Hooft, W.F., Heitkönig, I.M.A., Prins, H.H.T., Smidt, H. (2018) Occupancy strongly influences faecal microbial composition of wild lemurs (*Microbial Ecology* 2018: 94(3), accepted and in press, manuscript ID: EMI-2017-1072)

de Winter I.I. and Umanets A, Gort, G., Nieuwland, W., van Hooft, W.F., Heitkönig, I.M. A., Kappeler, P.M., Prins, H.H.T., Smidt H. Effects of seasonality and previous logging on faecal helminth-microbiota associations in wild lemurs (*Scientific Reports*, under review, manuscript ID: SREP-17-16226A)

de Winter, I.I., Qurkhuli, T., de Groot, N, van Hooft, W.F., Heitkönig, I.M.A., Prins, H.H.T., Doxiadis, G. Unravelling the *Mhc-DRB* profile of three congeneric brown lemur species in wild populations by non-invasive methods (Submitted to *Immunogenetics* (IMMU-D-18-00068))

de Winter, I.I., van Hooft, W.F., Koene, P., Larney, E and Wright, P. C. The coexistence of two congeneric lemur species. (*International Journal of Primatology*, under review, manuscript ID: IJOP-D-18-00024, presented at the 18th Benelux congress of Zoology and documented in *Bionieuws*, Vol. 6)

de Visser, M.C., de Winter, I.I., Van Hooft, W.F., Matson, K.D., Bionda, T. Stress in captive blue-eyed black lemurs (*Eulemur flavifrons*) (*Zoo Biology*, under review, manuscript ID: ZOO-17-121)

Podgorskia, I.I., Pantóa, L., Földesa, K., de Winter, I.I., Jánoskaa, M., Baptiste Chenet, E.S., Benkőa, M., Harracha, B. Newly detected adenoviruses in representatives of the most ancient primate lineages support the theory on hostvirus co-evolution (submitted to *Infections, Genetics and Evolution*, manuscript ID: MEEGID-D-17-00782)

de Winter, I.I. de, Heitkönig, I.M.A., van Hooft, W.F., Wright, P. C, Prins, H.H.T. (2014). Parasite prevalence in lemurs: Habitat disturbance and individual characteristics as explaining factors. Proceedings of the 83rd Annual Meeting of the American Association of Physical Anthropologists - *American Journal of Physical Anthropology* 153: 275 – ISSN 0002-9483

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de Winter, I.I., Heitkönig, I.M.A., Wright, P.C., Prins, H.H.T. (2013). Individual variation in parasite infections in lemurs. Contrib. to proc: International Conference on Individual Differences, 20th KNDV Zoology Conference, 1-3 Nov 2013, Groningen, The Netherlands - p. 130

de Winter, I.I., et al. Assessing the viability of blue-eyed black lemur subpopulations in a fragmented landscape (in preparation, expected submission date: Oct. 2018)

de Winter, I.I., et al., Helder, H., van Elsen, S., et al. Genetic identification of gastro-intestinal parasites in wild *Eulemurs* from different geographic regions in Madagascar (in preparation, expected submission date: autumn 2018)

de Winter, I.I. , Houtman, N. Nieuwland, W, et al. Temporal and seasonal variation in parasite infection in *Eulemur* species (in preparation, expected submission date: autumn 2018)

PE&RC TRAINING AND EDUCATION STATEMENT

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- The impact of human-induced habitat changes and natural stress factors on lemur health

Writing of project proposal (4.5 ECTS)

- Parasite prevalence in wildlife: the effect of anthropogenic disturbances and environmental, ecological, and physiological factors from a multi-scale perspective (2012)

Post-graduate courses (7.1 ECTS)

- Introduction to R for statistical analysis; PE&RC (2012)
- Entrepreneurship in and outside Science (ES); WGS (2015)
- ESG Loopbaanbegeleiding (career guidance); WGS (2015, 2016)
- Career Perspectives (CP); WGS (2016)

Laboratory training and working visits (3.5 ECTS)

- DRB Diversity and gene duplication; Biomedical Primate Research Centre, Rijswijk (2012-2018)
- Constructive advice from wildlife veterinarian; Burgers' Zoo (2012-2016)
- Parasite isolation and identification course; Department of Infectious Diseases and Immunology, Utrecht University (2012-2016)
- Lemur Curator meetings; Apenheul, Apeldoorn and Zoo Dresden, Dresden (2012-2016)
- Primate behaviour; Department of Environmental Biology, Utrecht University (2012)

Deficiency, refresh, brush-up courses (3 ECTS)

- Training at SNP Natuurreizen, ANVR-certified tour manager (2016)

Competence strengthening / skills courses (3 ECTS)

- Personal leadership the choice; ESG (2014)
- Competence assessment; WGS (2013)
- Data management; WGS (2013)
- Scientific writing; WGS (2012, 2013)
- Reviewing a scientific paper (RSP); WGS (2012)

PE&RC Annual meetings, seminars and the PE&RC weekend (3 ECTS)

- PE&RC Day (2012-2017)
- PE&RC Weekend last stretch (2016)
- WGS PhD workshop carousel (2014, 2015)
- PE&RC Weekend (2012)

Discussion groups / local seminars / other scientific meetings (7.5 ECTS)

- WEES Lectures and workshops (2012-2018)
- NVG PhD Workshop and presentation lemur parasites; Soesterberg (2012)
- NAEM; poster presentation; NERN (2013-2016)
- Discussion group PE&RC: ecological theory and application (2012-2016)
- NAEM; poster presentation; NERN (2013-2016)
- 'R' User meetings (2012-2016)

International symposia, workshops and conferences (5.7 ECTS)

- 6th European Wildlife Disease Association (EWDA) Student Workshop on 'human drivers of emerging diseases'; oral presentation, Veyrier du Lac, France (2015)
- 5th Congress of the European Federation for Primatology (EFP): 'Primates in our hands'; oral presentation, Antwerp, Belgium (2013)
- Infectious disease workshop; oral presentation; Centre ValBio, Ranamafana, Madagascar (2013)
- Student conference on conservation science; poster presentation; Cambridge, UK (2014)
- 25th Congress of the International Primatological Society (IPS); oral presentation; Hanoi, Vietnam (2014)

Lecturing / supervision of practicals / tutorials (3 ECTS)

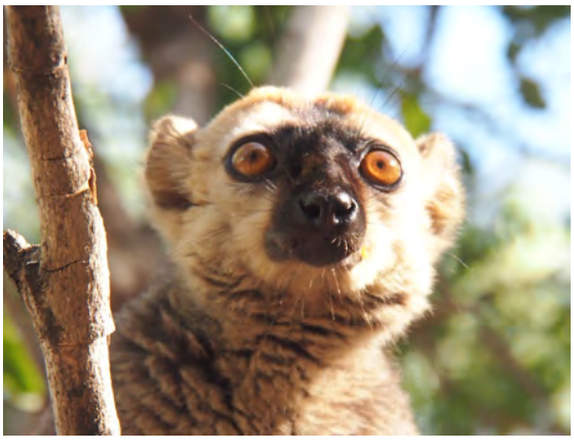
- Resource ecology (2013-2016)
- Ecological methods (2013-2016)

Supervision of 26 MSc students and 4 BSc within the PhD project

- 20 MSc Thesis students from universities within the Netherlands. Projects included fieldwork in Madagascar and lab work in the Netherlands
- 2 MSc Thesis students from the Université d'Antananarivo. Projects included fieldwork in Madagascar
- 4 MSc Students from universities within the Netherlands. Projects included lab work in the Netherlands
- 4 BSc Students from universities within the Netherlands. Projects included theoretical work in the Netherlands











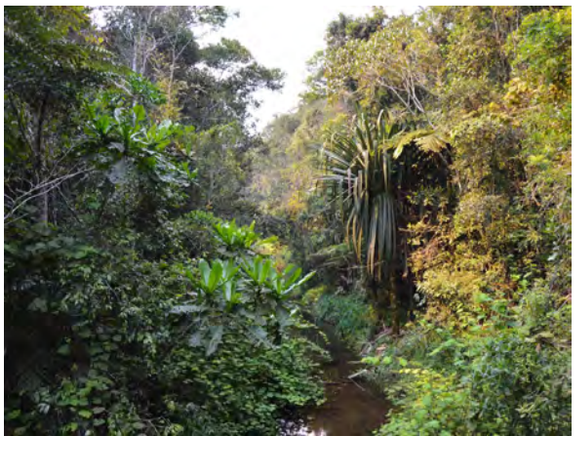










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