Investigations on the abundance of ectoparasites and vector-borne pathogens in southwest Madagascar

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Summary

Human encroachment on natural habitats is steadily increasing due to the rapid growth of the worldwide population. The consequent expansion of agricultural land and livestock husbandry, accompanied by spreading of commensal animals, create new interspecific contact zones that are major regions of risk of the emergence of diseases and their transmission between livestock, humans and wildlife. Among the emerging diseases of the recent years those that originate from wildlife reservoirs are of outstanding importance.

Many vector-borne diseases are still underrecognized causes of fever throughout the world and tend to be treated as undifferentiated illnesses. The lack of human and animal health facilities, common in rural areas, bears the risk that vector-borne infections remain unseen, especially if they are not among the most common. Ectoparasites represent an important route for disease transmission besides direct contact to infected individuals. While factors driving disease emergence are clearly visible in Madagascar, the knowledge of ectoparasites and potential pathogens is alarmingly limited.

Following the One Health concept, this dissertation approaches the presence of ectoparasites and vector-borne bacteria, taking environmental factors and host ecology into account in order to identify possible transmission paths at the human/animal interface. The study took place in the northern portion of Tsimanampetsotsa National Park and the adjacent coastal strip in southwest Madagascar, in the dry and in the rainy season of 2016/2017. For the survey on ectoparasites species and the influence of habitat alteration, endemic mammals were trapped in transects of box traps installed in three habitats revealing different degrees of disturbance: Forest of the national park, degraded forest, and cultivated land. Additionally, *Rattus rattus and Mus musculus* were trapped inside villages, and livestock was sampled.

I identified 17 species of ectoparasites, thirteen of which (blood-feeding lice, fleas and ticks) were subjected to further investigation for the presence and identity of rickettsiae, borreliae, bartonellae and *Yersinia pestis* using PCR techniques. This study presents the molecular detection of a broad spectrum of vector-borne bacteria including pathogens: *Yersinia pestis* in *X. cheopis*, *Ricksttsia felis*-like organisms in several fleas and *Rickettsia africae* in all ectoparasite species except one. Borreliae and bartonellae were found only in two ectoparasite species each. A large portion of the parasites was host-specific, barely stating threats for cross species transmission. Apart from the introduced flea species *Echidnophaga gallinacea* no interspecific parasite transfer occurred between introduced and native mammals. *Microcebus griseorufus*, which was the only endemic mammal caught in statistically sufficient numbers, revealed the highest louse prevalence inside the least degraded habitat, contrary to the common assumption that the prevalence of ectoparasites increases in degraded areas. This study also confirms the littoral site as less suitable habitat for most introduced ectoparasite species.

In a remote area with a poorly developed public health system and limited options for treatment, the knowledge of the presence of the detected pathogens and their possible vectors enables the population to preventive behavior. Therefore, this piece of research on the occurrence of pathogens is of outstanding relevance to the local health care, as it reveals potential threats of transmission of pathogenic *Y. pestis* or *R. africae* at the human/animal interface.

Zusammenfassung

Anthropogene Eingriffe in natürliche Lebensräume nehmen aufgrund des starken Wachstums der Weltbevölkerung stetig zu. Durch die daraus resultierende Ausdehnung der landwirtschaftlichen Nutzflächen, die Zunahme der Tierhaltung und die gleichzeitige Ausbreitung kommensaler Arten werden neue interspezifische Kontaktzonen geschaffen, die bedeutende Risikogebiete für das Auftreten von Krankheiten und deren Übertragung zwischen Nutztieren, Menschen und Wildtieren darstellen. Die neu auftretenden Krankheiten der letzten Jahre, die aus Wildtier-Reservoirs hervorgingen, sind von herausragender Bedeutung.

Viele Vektor-übertragene Krankheiten sind weltweit immer noch vernachlässigte Ursachen für Fieber und werden tendenziell als undifferenzierte Krankheiten behandelt. Der Mangel an Gesundheitseinrichtungen für Mensch und Tier, wie er in ländlichen Gebieten häufig vorkommt, birgt das Risiko, dass durch Vektoren übertragene Infektionen unbemerkt bleiben, insbesondere wenn sie nicht zu den am häufigsten auftretenden gehören. Ektoparasiten stellen neben dem direkten Kontakt zu infizierten Individuen einen wichtigen Übertragungsweg für Krankheiten dar. Während die Faktoren, die das Auftreten von Krankheiten begünstigen, in Madagaskar deutlich sichtbar sind, ist das Wissen über Ektoparasiten und potenzielle Krankheitserreger alarmierend gering.

Im Sinne des One Health-Konzepts befasst sich diese Dissertation mit dem Vorkommen von Ektoparasiten und Vektor-übertragenen Bakterien unter Berücksichtigung von Umweltbedingungen und der Ökologie der Wirtstiere, um mögliche Übertragungswege an der Schnittstelle zwischen Mensch und Tier zu identifizieren. Die Studie fand im nördlichen Teil des Tsimanampetsotsa-Nationalparks und im angrenzenden Küstenstreifen des Südwestens Madagaskars in der Trocken- und Regenzeit 2016/2017 statt. Zur Untersuchung von Ektoparasitenarten und von Einflüssen der anthropogenen Veränderung des Lebensraums wurden endemische Säugetiere mit Lebendfallen in Transekten gefangen, die in drei Lebensräumen mit unterschiedlichem Störungsgrad installiert wurden: Im Wald des Nationalparks, in degradiertem Wald und in Kulturland. Zusätzlich wurden *Rattus rattus* und *Mus musculus* in Dörfern gefangen, und Nutztiere untersucht.

Ich identifizierte 17 Arten von Ektoparasiten, von denen dreizehn (blutsaugende Läuse, Flöhe und Zecken) unter Verwendung von PCR-Techniken auf das Vorkommen von Rickettsien, Borrelien, Bartonellen und *Yersinia pestis* untersucht wurden. Diese Studie präsentiert den molekularen Nachweis eines breiten Spektrums von durch Vektoren übertragener Bakterien, einschließlich mehrerer Krankheitserreger: *Yersinia pestis* bei *X.*

cheopis, *Ricksttsia felis*-ähnliche Organismen bei mehreren Floharten und *Rickettsia africae* - mit einer Ausnahme - bei allen Ektoparasitenarten. Borrelien und Bartonellen wurden nur in jeweils zwei Ektoparasitenarten gefunden. Ein großer Teil der Parasiten war wirtsspezifisch und stellt kaum eine Gefahr für die Übertragung zwischen verschiedenen Arten dar. Abgesehen von der eingeschleppten Flohart *Echidnophaga gallinacea* trat keine interspezifische Übertragung von Parasiten zwischen eingeschleppten und endemischen Wirtsarten auf. *Microcebus griseorufus* ist die einzige Wirtsart, die in statistisch ausreichender Anzahl gefangen wurde. Dieser Wirt zeigte die höchste Lausprävalenz innerhalb des am wenigsten degradierten Lebensraums, entgegen der allgemeinen Annahme, dass die Prävalenz von Ektoparasiten in degradierten Gebieten zunimmt. Diese Studie bestätigt auch die Küstenregion als weniger geeigneten Lebensraum für die meisten eingeschleppten Ektoparasitenarten.

Für ein abgelegenes Gebiet mit einem schlecht entwickelten öffentlichen Gesundheitssystem und begrenzten Behandlungsmöglichkeiten ermöglicht die Kenntnis über das Vorkommen der hier nachgewiesenen Krankheitserreger und ihrer möglichen Vektoren, dass sich die Bevölkerung präventiv verhalten kann. Daher ist diese Arbeit über das Auftreten von Krankheitserregern für die lokale Gesundheitsversorgung von hohem Stellenwert, da sie potenzielle Bedrohungen für die Übertragung der Pathogene *Y. pestis* oder *R. africae* zwischen Mensch und Tier aufdeckt.

Chapter 1

General introduction

Human alteration of natural habitats is one of the major drivers of wildlife disease outbreaks (Daszak et al., 2000; Dobson and Foufopoulos, 2001). "Pathogen pollution" meaning the introduction of diseases and parasites to wildlife (Cunningham et al., 2003) has caused heavy decline in animal populations (Chapman et al., 2005; Estrada et al., 2018; Köndgen et al., 2008). Reverse directed transmissions of new human pathogens from wildlife reservoirs, demonstrated by emerging infectious diseases like Ebola, AIDS, Plague, SARS or COVID-19 have accounted for some of the most devastating epidemics and pandemics (Burki, 2017; Dong et al., 2020; Drosten et al., 2003; Hahn et al., 2000; Leroy et al., 2009). These rely on human-wildlife interactions driven by the extension of contact zones, which open the door for pathogen transmission from wildlife to humans and vice versa (Daszak et al., 2000; Gortazar et al., 2014). Besides direct contact to body fluids of infected individuals for example through bushmeat hunting and butchering (Wolfe et al., 2005), ectoparasites, hence termed vectors, can mediate cross-species transmission of pathogens (Gortazar et al., 2014).

The awareness that anthropogenic disturbance of ecosystems has a profound effect on disease dynamics crucially developed in the last two decades, and is usually propagated with the term One Health in recent days (Kahn et al., 2008). One Health attempts to recognize the unquestionable interplay between the health of humans, animals and their shared environment The American Veterinary Medical Association defined that "One Health is the collaborative effort of multiple disciplines – working locally, nationally, and globally – to attain optimal health for people, animals and our environment" (AVMA, 2008). Simultaneously the discipline of conservation medicine emerged, which discovers, according to the concept of One Health, the connection between changes of habitat quality, emerging pathogens, health of animal communities and humans, but emphasizing the importance of maintaining biodiversity and ecosystem functions. Conservation medicine considers the health aspect of biodiversity conservation (Aguirre et al., 2012).

Early endeavors to promote a closer collaboration between human medicine and veterinary sciences were made by the German physician Robert Virchow, who stated that human and animal health are inextricably connected and created the term "zoonosis" (Kahn et al., 2007), which means a human disease of animal origin. Virchow's approach was taken up by Schwabe (1984), whose coining of the term "one medicine" was an important step towards One Health and conservation medicine from the human health perspective. By considering ecological and environmental features in matters of health sciences the ideas behind ecohealth can be understood as the component of conservation medicine from the ecology side. The central point of ecohealth is that health and disease are fundamentally related to the integrity of ecosystems (Schaeffer et al., 1988; Tabor, 2002; Wilcox et al., 2012). Biodiversity is substantial for an intact ecosystem including the preservation of ecosystem functions and ecosystem services (Gascon et al., 2015). The loss of biodiversity is connected to ecosystem change accompanied by decrease in benefits for humanity (Cardinale et al., 2012; Hooper et al., 2012).

Regarding vector-borne diseases, biodiversity provides an important ecosystem service (Keesing and Ostfeld, 2012). A diverse host fauna can reduce pathogen distribution and transmission by, firstly, reducing encounter rates between infected hosts and the vector, or in the case of host specific parasites, simply by confining the host abundance by competition or predation. Secondly, transmission is reduced by the host encountered by the vector being less susceptible for the pathogen than others, and thirdly, by decreasing the relative abundance of susceptible hosts (Keesing et al., 2006). The last two points refer to the dilution effect (Ostfeld and Keesing, 2000b), a phenomenon that has been observed in directly transmissible rodent-borne diseases (Carver et al., 2011), but most thoroughly studied for Lyme disease (LoGiudice et al., 2003; Ostfeld and Keesing, 2000a)

The dilution effect sets in for the case that the vector is a generalist parasite on different hosts. In the example of Lyme disease, ticks readily get infected by feeding on certain species of small mammals, while the efficiency of *Borrelia* acquisition is much lower when feeding on carnivores, ungulates or songbirds. This host-dependent ability of infecting the vector by the blood meal is called reservoir competence (Ostfeld and Keesing, 2012). Given that acquisition of pathogens strongly depends on the source of blood meal, the average reservoir competence of hosts is reduced in a diverse host community compared to a community of poor biodiversity. A less diverse host community, which is dominated by a highly competent reservoir host feeding the greatest proportion of the vector population, would lead to a significantly higher pathogen prevalence and transmission rate (Chivian and Sullivan, 2002; Norman et al., 1995; Ostfeld and Keesing, 2000b). In other words, a high diversity of hosts may dilute a pathogen in a reservoir pool and decrease the possibility that the vector will become infected.

Host population densities pose a key factor for parasite and disease transmission, given the frequently observed link of higher ectoparasite and pathogen prevalence to higher host densities (Anderson and May, 1979; Lafferty and Holt, 2003; Lindenfors et al., 2007; Mbora and McPeek, 2009; Stanko et al., 2002) Habitat fragmentation stimulates this relation as densities tend to rise in fragments (Debinski and Holt, 2000; Suzán et al., 2012). Moreover, fragmentation can add another factor supporting parasite abundance, because on the individual level hosts can suffer physiological stress resulting in increased susceptibility to parasite infestation (Irwin et al., 2010; Junge et al., 2011). In ticks, higher host abundances raise the chances to encounter hosts to complete their life cycle, which leads to a growing tick population (Randolph, 2004).

In Madagascar ecosystems are affected by human activities in many ways. Driven by the rapidly growing human population (UNFPA, 2019) deforestation and habitat fragmentation are outstanding (Harper et al., 2007). About 85 % of the population lives in rural areas (Scales, 2014) where the demand for limited natural resources and space for agriculture is already high, but still increasing due to population growth. Timber is extracted for the construction of houses. Dead wood is collected for cooking or, alternatively, charcoal is used, the production of which is also a major cause of deforestation. Agricultural fields replace wooded areas and often arise from the unsustainable slash and burn procedure. Nearly half of the spiny forest of the south west was lost in the past 40 years due to human alteration (Brinkmann et al., 2014; Casse et al., 2004). The dry forest of south-western Madagascar has been reduced and degraded substantially by agriculture and grazing by goats and cattle (Brinkmann et al., 2014; Ratovonamana et al., 2013; Zinner et al., 2014). In this region of Madagascar the human dependence on forest products and the pressure on the remaining forest habitats are particularly high (Andriamparany et al., 2014; Neudert et al., 2015). As one of the driest regions of Madagascar the annually precipitation rarely exceeds 400 mm and rain falls usually from December to April, but very irregularly, and some years are completely lacking rainfall (Ratovonamana et al., 2013). Especially in the South cattle herding has a big local value not only as food insurance when crop yield fails in periods of drought, but also as a symbol of wealth, social status and as capital reserve (Goetter, 2016).

As an "area featuring exceptional concentrations of endemic species and experiencing exceptional loss of habitat" Madagascar is categorized as a biodiversity hotspot and emphasized for global conservation action (Myers et al., 2000). Estimated 95% of reptiles, 99% of amphibians and 100% of the land mammals in Madagascar exist nowhere else in the world (Yoder and Nowak, 2006). The local endemism in the southwest is equally high. The regional vegetation forms the characteristic spiny and dry deciduous forest, globally unique in terms of structure and taxa (Olson and Dinerstein, 2002). Forest destruction has already caused measurable changes in mammal communities in many places of Madagascar (Irwin et al., 2010; Scott et al., 2006). The endemic mammal fauna of Madagascar evolved in the isolation of an island, and may be more susceptible to novel pathogens which they have no immune competence for (De Castro and Bolker, 2005). The impact of invasive species can also be induced by the interplay with parasites. The invasive species cannot only transport parasites that may infect the local species, but they can also serve as new hosts for parasites or as reservoir for pathogens already present (Cleaveland et al., 2002; Daszak et al., 2000). It is suggested that fragmented habitats are particularly prone to invasions of both invasive species and infectious diseases (Suzán et al., 2012), which applies to some incidences of pathogen pollution in Madagascar (Bublitz et al., 2015; Junge et al., 2011).

Figure 1.1: Endemic mammals of the spiny forest. Gray-brown mouse lemur, *Microcebus griseorufus* (left); lesser hedgehog tenrec, *Echinops telfairi* (top right); giant-striped mongoose *Galidictis grandidieri* (bottom right).

There are six species of endemic small mammals occurring in the study area. Four species of tenrecs (Tenrecinae) are represented by the lesser hedgehog tenrec (*Echinops telfairi*), the greater hedgehog tenrec (*Setifer setosus*), the common tenrec (*Tenrec ecaudatus*) and the large-eared tenrec (*Geogale aurita*). The two endemic rodents (Nesomyinae) are the western tuft-tailed rat (*Eliurus myoxinus*) and the western big-footed mouse *Macrotarsomys bastardi* (Soarimalala and Goodman, 2011). The giant-striped mongoose (*Galidictis grandidieri*) and the fossa (*Cryptoprocta ferox*) are the only representatives of endemic carnivores (Eupleridae) reported (Sauther et al., 2013; Ratovonamana, pers. comm.). Three lemur species appear in the proximity: The grey-brown mouse lemur (*Microcebus griseorufus*), the ring-tailed lemur (*Lemur catta*) and sporadically Petter's sportive lemur (*Lepilemur petteri*). They all not only have to cope with the unpredictable and harsh climatic and environmental conditions, but also with the influences of invasive species. House mice (*Mus musculus*), black rats (*Rattus rattus*), wild cats (*Felis sylvestris*) and bush pigs (*Potamochoerus larvatus*) extend the regional wild mammal fauna as introduced portion (Sauther et al., 2013; pers. obs.).

Most momentously, black rats have been introduced to Madagascar roughly between the 11th and 14th century as human commensals on ships (Lehtonen et al., 2001). Invasion into the most remote parts of Madagascar may have occurred not until the 20th century (Hingston et al., 2005), however, the distribution of the black rat has nowadays expanded over the entire island (Raharivololona et al., 2007). Rats are not only dwelling in human housings but also enter natural habitats (Ganzhorn, 2003; Goodman, 1995). Their generalist lifestyle facilitates movement patterns between pristine and disturbed habitats, making them a potential carrier of parasites and pathogens into former unaffected areas (Ellis et al., 1999; Smith and Carpenter, 2006). Rats have been shown to share many parasites with endemic small mammals and with lemurs as well (Raharivololona et al., 2007). Generalist introduced rodents seem to cope better than native species with disturbed habitats as they tend to exhibit higher population densities in secondary forest (Ganzhorn, 2003; Lehtonen et al., 2001). Apart from competition and predation rats can transmit diseases with devastating result to the native mammal populations (MacPhee and Marx, 1997). By harboring the causative agents of plague (*Yersinia pestis*), murine typhus (*Rickettsia typhi*) or salmonellosis (*Salmonella enterica*) rats pose a significant threat not only to wildlife but also to human health in Madagascar (Duplantier and Duchemin, 2003). Similar to rats, stray dogs and feral cats represent potent bridging species. They may play a peculiar role as hosts for ectoparasites of different domestic animals and wild animals, as they reveal an explicit roaming behavior promoting contact with different animals and therefore acquisition of different parasites.

Constituting another source of pathogen pollution, in the course of livestock importation, parasites and pathogens are introduced to an unknown extent. For instance, ticks of cattle have spread around the globe through animal trade and also reached Madagascar (Barre et al., 1995; Barre and Uilenberg, 2010; Uilenberg, 1964). As a part of the traditional livestock husbandry, cattle are driven over large distances heading for food and water sources in many parts of Madagascar thereby crossing both degraded and protected areas. Apart from that, a common practice of husbandry is driving livestock for pasture into forests on purpose

(Feldt and Schlecht, 2016; Goetter, 2016; Ratovonamana et al., 2013). Cattle supposedly hold a big potential to both spread parasites to and receive pathogens from the invaded habitats. Minimizing that risk, however, is a big effort because of the local status hold by the livestock sector. Cattle are not only food reserve, but the main source of capital and social status in the southwestern region. Deeply rooted in tradition and culture, livestock constitutes the fundamental form of material wealth (Fauroux, 1997; Goetter, 2016).

The lifestyle of many pastoralists not only gets along with close contact to livestock, but also with exposure to endemic fauna during movement through habitats of mammals and parasites. The following section provides general information about ectoparasites and introduces three of their most important genera as subjects of this thesis.

Parasites play an important role in ecosystem functioning as they provide the ecosystem service of regulating wildlife populations (Hudson et al., 2006; May, 1983; Tompkins and Begon, 1999). A large proportion of all species on earth is represented by parasites, which is why they are regarded as a driving force in ecology and evolution. On a local scale they are an important selective force on populations (Dobson et al., 2008; Poulin and Morand, 2000). One the other hand, from the human perspective, they can be responsible for economical losses in animal husbandry and have a huge impact on public health. By their blood feeding behavior they can directly harm the host by causing anemia and inflammatory responses, and secondly, they are able to transmit diseases (Goddard, 2009).

Parasitism is a type of symbiotic relationship between two organisms where one is living at the other's expense. In this symbiosis the relationship is harmful to the organism providing resources, called the host. The organism which exploits the host as a source of nutrition and as a habitat is the parasite (Combes, 2001). While the parasite profits, the host may suffer substantially from pathological damage, decreased fitness and reduced survival (Delahay et al., 1995; Milton, 1996). The majority of ectoparasites - despite some blood-sucking leeches - belong to the phylum of arthropods (Hopla et al., 1994). In contrast to endoparasites, which absolutely rely on the host for survival, ectoparasites usually establish a temporary relationship to their host meaning that they are also viable off-host to a certain degree. Ectoparasites reveal a broad range of impact to the host's health. Some only feed on skin debris or hair but can irritate the skin with their excrement, while the female jiggers (*Tunga penetrans* fleas), for example, spend their life burrowed in the host's skin. Some Diptera lay eggs into the host's skin where then larval development takes place (Goddard, 2009). There is a variety of ectoparasite species from different arthropod taxa, which are specialized on feeding on the blood of vertebrates. Most common ectoparasites of mammals, besides the much noticed winged parasites like mosquitoes, belong to the orders of Ixodida (ticks), Phthiraptera (lice) and Siphonaptera (fleas). Blood sucking ectoparasites obtain special attention for their ability to host and transmit pathogens (Lehane, 2005).

Parasites reveal complex life cycles and the parasite-host interplay is prone to life history traits and population dynamics of the hosts and to environmental factors like habitat structure and climatic conditions (Altizer et al., 2006; Tukahirwa, 1976). Temperature and humidity affect development and life-cycle completion times as well as survival rates (Harvell et al., 2002; Ostfeld, 2009; Plowright et al., 2012). Higher temperatures tend to increase energy demand resulting in higher biting rates (Plowright et al., 2012). For generalist parasite species the source of female adult's blood meal can affect egg survival and reproductive success (Dipeolu and Akinboade, 1984; Krasnov et al., 2004).

The order of Ixodida (ticks) are assigned to the subclass Acari, like mites. According to the latest comprehensive review by Guglielmone et al. (2010) 896 tick species divided into three families have been described. The three families comprise the Ixodidae (702 species), also called hard ticks characterized by their sclerotic scutum, the Argasidae (193 species) or soft ticks, so called because of their flexible cuticle, and the Nuttalliellidae (1 species), which are regarded as the basal family (Sonenshine and Roe, 2013b). Ticks are obligate blood-sucking ectoparasites to every vertebrate group of Tetrapoda. For taking the blood meal ixodid ticks may stay attached to the host for some days up to several weeks (when infesting reptiles) (Apanaskevic and Oliver, 2013). Their special retroserrated mouthparts and secretion of cement facilitate the long lasting attachment (Alarcon-Chaidez, 2013; Sonenshine and Anderson, 2013).

The life cycle of ixodid ticks comprises the egg and three active stages, namely, larva, nymph and adult. There is only one blood meal between the life stages. Typically each life stage of ixodid ticks feeds on a single host, and larvae and nymphs drop off the host and molt to turn into the next stage after a blood meal, thus having a three-host life cycle (Figure 1.2). There are also one-host and two-host life cycles, where the larvae or both, larvae and nymphs, do not drop off, but remain and develop on the host (Apanaskevic and Oliver, 2013). The female adults gain up to 100 times of their original weight during engorgement and lay thousands of eggs in a sheltered environment after dropping off the host. Following oviposition the female dies. Ixodid ticks spend more than 90% of their lives off the host (Needham and Teel, 1991) and the entire life cycle may last for several years. In their off-host environment they depend on a certain microclimate with narrowly defined temperature and humidity requirements (Lees, 1946; Londt and Whitehead, 1972). The main difference in the development of argasids is the so-called multiple-host life cycle as there are multiple nymphal stages and female adults feed and oviposit several times. Argasid ticks feed only for short time periods, usually 15 to 30 minutes. (Apanaskevic and Oliver, 2013; Parola and Raoult, 2001).

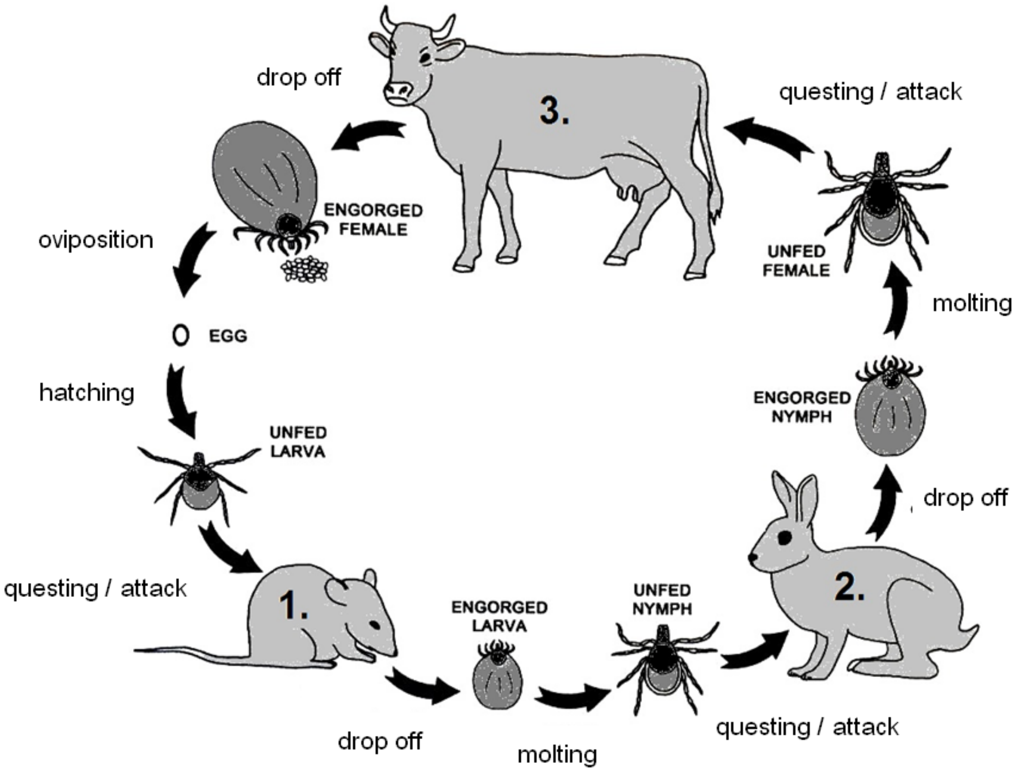

Figure 1.2: Three-host life cycle of Ixodidae ticks (modified from Apanaskevic and Oliver, 2013).

Tick species differ greatly in host ranges. Some live closely related to the burrows or nests of their hosts like rodents, birds or bats. These tick are often but not necessarily host specialists. Whereas *Ixodes ricinus*, for example, is found in open vegetation, questing for and parasitizing small mammals, lizards or ground-dwelling birds, as well as medium- and large-sized mammals (Sonenshine and Roe, 2013a). Ticks are major vectors of zoonotic diseases, transmitting the greatest diversity of pathogenic agents prior to mosquitoes. Pathogens transmitted by ticks include viruses, bacteria, funghi, helminths and protozoans. Predominantly known for transmitting Lyme disease in the northern temperate zones and tick-borne encephalitis in Europe, ticks are also vectors of several spotted fever rickettsioses or heart water disease and anaplasmoses of ruminants. (de la Fuente et al., 2008; Pfäffle et al., 2013).

The order of Siphonaptera (fleas) comprises around 2500 species of fleas distributed over 16 families (Whiting et al., 2008)**.** Fleas are holometabolous wingless insects, the imagines of which are blood feeding on mammalian and avian vertebrates. Their laterally flattened body bearing features like setae and combs with specific numbers of spines adapts them to fur or feathers (Marshall, 1981). Fleas hold an important role as vectors of a variety of pathogens for humans and animals, most notably being responsible for transmission of the plague bacterium *Yersinia pestis*. According to their life history fleas can be distinguished between "body fleas" and "nest fleas". "Body fleas" spend most of their life on a host, whereas the "nest fleas" spend their life mainly in the host nest or burrow and visit the host only to feed. The majority of fleas parasitize hosts that offer such shelters (Krasnov, 2008b). Adult fleas feed several times during the day with a temperature-dependent frequency of blood meals, peaking at a distinct species-specific optimum, and decreasing when temperature is lower or higher (Krasnov, 2008a). Fleas are able to endure several months of starvation while their host animal is absent. Albeit distinct host preferences, fleas will feed on other animals when the major host is absent (Lehane, 2005).

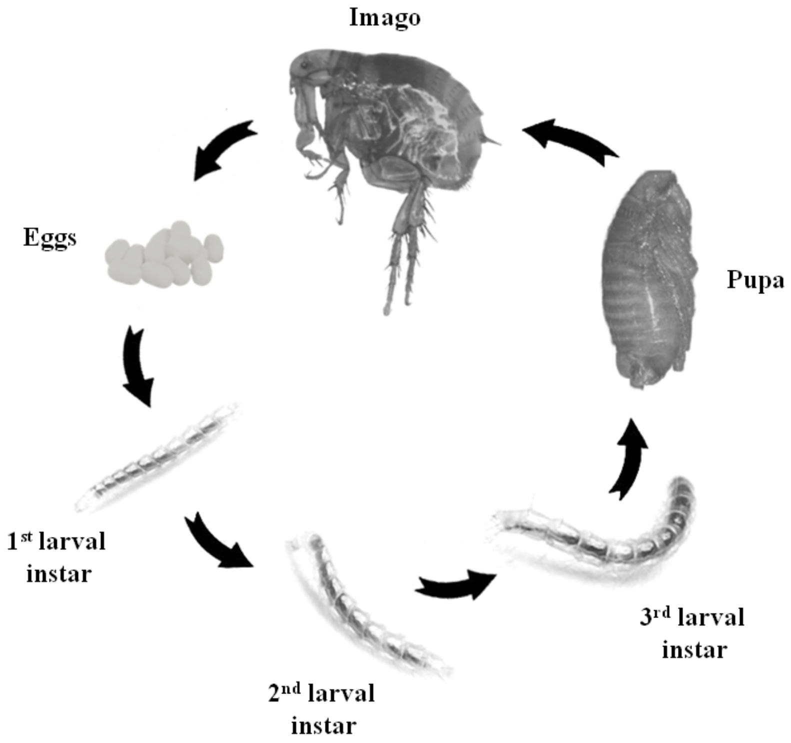

Figure 1.3: Schematic life cycle of fleas.

Female fleas lay up to 25 eggs per day on and off-host. Three maggot-like larval stages last about 2 to 3 weeks, followed by the third stage larva spinning a cocoon for the pupa stage (Dobler and Pfeffer, 2011; Figure 1.3). Pupae may persist for a longer time, and adult emergence is induced by environmental factors like vibration, increasing temperature or CO_2 concentration (Cotton, 1970; Krasnov, 2008c; Marshall, 1981). Fleas feeding on bacteremic vertebrate hosts can acquire pathogens which proliferate inside the flea. Flea-borne infection of vertebrates through contaminated feces is the common mode of transmission of rickettsiae and bartonellae, which proliferate inside the midgut (Chomel et al., 2009). Cat fleas, for example, have been shown to excrete infective *R. typhi* for up to ten days (Azad and Beard, 1998). The feces are an important source of nutrition for the larvae of some species, which can readily get infected by feeding (Reif and Macaluso, 2009). Vertical transmission of rickettsiae within flea populations has also been described (Farhang-Azad et al., 1985). Y*ersinia pestis* transmission to the host via flea bites is particularly due to its predominant development inside the flea's foregut (Gage, 2012).

Lice (Phtiraphtera) are dorsoventrally flattened and wingless hemimetabolous insects. As obligatory parasites of birds and mammals they are totally dependent on their host and adapted to its skin fur/feather environment (Lehane, 2005). The order of Phtiraphtera comprises the suborders Anoplura, Rhyncophthirina, Amblycera and Ischnocera. Lice tend to be highly host specific, parasitizing only one distinct species or a few closely related species at the most (Price and Graham, 1996). Anoplura are called sucking lice as they feed exclusively on blood (Kim, 2006). They are found only on mammals. Two thirds of over 500 louse species are associated with rodents (Price and Graham, 1997) and 20 are parasites of domestic animals or humans (Hopla et al., 1994). The suborders Amblycera (some 3,000 species) and Ischnocera (some 300 species) are ectoparasites of both birds and mammals. Due to their feeding specialized on keratinous substances of feathers, hairs, and skin debris they are called chewing lice (Price and Graham, 1997). Only three species belong to the suborder Rhynchophthirina, which are blood-sucking ectoparasites of Elephants, warthogs and red river hogs, respectively, in Africa (Hellenthal and Price, 2009)

Lice are not capable of starving for longer periods and need to feed several times a day. Anopluran lice die within two days without accomplishing blood intake (Lehane, 2005). Humidity and temperature are also critical factors for louse survival, which are susceptible to rapid dehydration. At optimal conditions (29 to 32°C and 70 to 90% relative humidity) an adult body louse usually lives 20 to 30 days and females produce offspring daily (Raoult and

Roux, 1999). The eggs are glued firmly to the hair shaft or feathers (the human body louse uses fibers of clothes). Nymph hatching lasts, temperature-dependent, about 1 week. The nymphs pass through three instars to adults in about two weeks (Lehane, 2005; Figure 1.4).

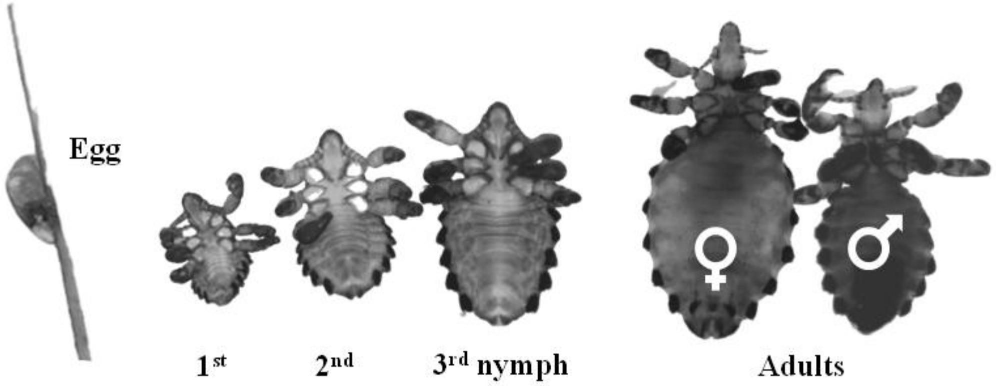

Figure 1.4: Life cycle of lice

Body lice get infected with pathogens in course of the meal on infected humans. The pathogens are either released in feces (*B. quintana*, *Rickettsia prowazekii*), or when the louse is crushed (*Borrelia recurrentis*). This vectorial capacity has been validated only for the body louse and the three mentioned pathogens (Fournier et al., 2002; Raoult and Roux, 1999). But lice are suspected to serve as vector for several other diseases, mostly bartonelloses (Gutierrez et al., 2014; Reeves et al., 2006), but also plague (Ayyadurai et al., 2010; Drali et al., 2015).

The following paragraphs present bacteria which are commonly vectored by ticks, lice and fleas. *Rickettsia* spp., *Borrelia* spp. and *Bartonella* spp. are associated with a constantly increasing number of diseases and tend to infect a broad variety of mammals. When investigating vector-borne bacteria in Madagascar one cannot evade *Y. pestis* as the agent of plague, which was consequentially included in the study.

Rickettsiae are Gram-negative alpha-proteobacteria in the order Rickettsiales. The genus *Rickettsia* reveals a cosmopolitan and extraordinary diverse range of hosts found in terrestrial, marine and freshwater habitats (Weinert et al., 2009). Its distribution within the phylum of arthropods is accordingly broad. Rickettsiae are obligate intracellular endosymbionts, often living in mutuality with their invertebrate hosts but also as true parasites capable of reproductive manipulation or even killing the host (Perlman et al., 2006). Rickettsiae thriving in bloodsucking arthropods can also be transmitted to vertebrate secondary hosts during the blood meal and cause disease. Other rickettsial species are excreted with the vectors' feces, and enter the host transdermally through the lesions of the biting sites or by inhalation. Arthropod vectors for these vertebrate pathogens tend to be insects (fleas and lice) or acarines (mites and ticks) (Gillespie et al., 2008).

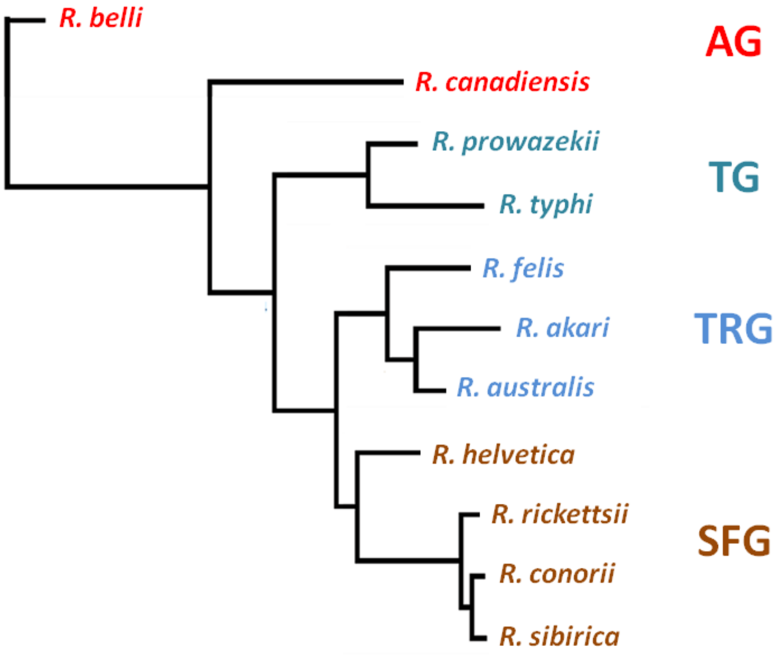

Figure 1.5: Phylogenetic tree of the genus *Rickettsia* showing the separation into four clades, namely the ancestral group (AG), the typhus group (TG), the transitional group (TRG) and the spotted fever group (SFG) (modified from Gillespie et al., 2008).

The different ways of transmission and the relation to different host taxa are reflected by the phylogeny of the genus. The pathogenic rickettsiae are grouped in four main clades, namely the ancestral group, the spotted fever group, the typhus group and the transitional group (Figure 1.5). The ancestral group is associated with ticks, and the transitional group infects both acarine and insect vectors. However, the most important rickettsial pathogens belong to the typhus and the spotted fever group. (Gillespie et al., 2008; Weinert et al., 2009). The two affiliates of the typhus group, *Rickettsia typhi* and *R. prowazekii*, the agents of typhus (murine typhus and epidemic typhus, respectively), are transmitted by insects. *R. typhi* is transmitted by fleas, usually *Xenopsylla cheopis* (Azad, 1990), and *R. prowazekii* is transmitted by body lice. These two pathogens are transmitted via contaminated insect feces (Parola et al., 2005)

Most of the *Rickettsia* pathogenic to humans are transmitted by hard ticks (Parola et al., 2005). Among these, a variety cause different forms of spotted fever (spotted fever group). Common symptoms are fever, headache, rash, and eschar development at the site of inoculation (Socolovschi et al., 2012). Human diseases like Rocky Mountain spotted fever (*R. rickettsii*), Mediterranean spotted fever (*R. conorii*) or African tick bite fever (*R. africae*) are examples for rickettsioses where vector and pathogen are known (Jensenius et al., 2003; Macaluso and Azad, 2005). However, there are many species with (yet) unexplained pathogenicity and also species which are found to be pathogenic, but only long after their discovery (Perlman et al., 2006). A peculiar characteristic of the tick-*Rickettsia* relationship is the maintenance of the rickettsiae by vertical (i.e. transovarial) transmission described for some species (Burgdorfer and Varma, 1967; Socolovschi et al., 2009), which may promote high *Rickettsia* prevalence in ticks (e.g. Ehlers et al., 2016; Keller et al., 2016).

Borreliae are Gram-negative spirochaetes in the order Spirochaetales. The genus *Borrelia* is well known for including *B. burgdorferi* s.l., the causative agents of Lyme disease in the northern hemisphere. In Africa, relapsing fever (RF) is the most frequent bacterial disease (Trape et al., 2013). Tick-borne relapsing fever is caused by several *Borrelia* species usually transmitted by soft ticks of the genus *Ornithodoros*. Three pathogenic *Borrelia* species of the RF-group are known from hard ticks, namely, *B. theileri* in *Rhipicephalus* sp., *B. miyamotoi* in *Ixodes* sp. and *B. lonestari* in *Amblyomma* species (Talagrand-Reboul et al., 2018). The louse-borne variant of relapsing fever is the epidemic relapsing fever, an infection with *B. recurrentis* transmitted to humans by body lice. Epidemic RF seems to be regularly emerging in continental Africa (Elbir et al., 2013). A third group of *Borrelia* transmitted by hard ticks is associated with reptiles (Takano et al., 2011; Takano et al., 2010; Figure 1.6).

The main and characteristic symptom of relapsing fever borreliosis is recurrent fever episodes of several days interrupted by afebrile periods. The fever is accompanied by violent pains of head, muscles and joints (Robert-Koch-Institut, 2011). *Borrelia* species which have a disease association with livestock (cattle) are *B. theileri* (*Rhipicephalus* spp.) (McCoy et al., 2014; Smith et al., 1978) and *B. coriaceae* (*Ornithodoros coriaceus*). Bovine borreliosis is a systemic disease which can affect the joints and show neurologic, renal and cardiac involvement (Hurst, 2018).

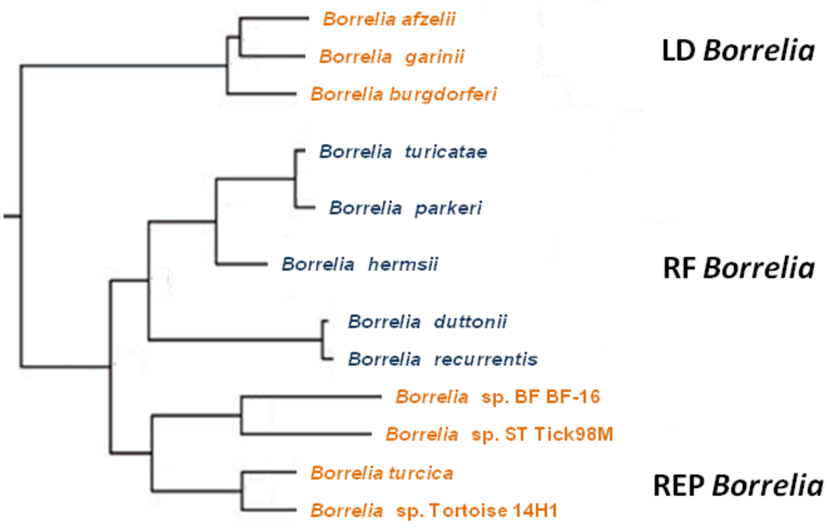

Figure 1.6: Phylogenetic classification of *Borrelia*. The genus splits up into three main groups: The Lyme disease group (LD), the relapsing fever group (RF) and the reptile-associated group (REP) (adapted from Takano et al., 2010).

Bartonellae belong to a genus of Gram-negative facultative intracellular alpha-proteobacteria. At least 13 human pathogens have been identified within the genus *Bartonella*, three of which cause the most relevant bartonelloses in humans: *B. bacilliformis*, *B. quintana* and *B. henselae* (Kaiser et al., 2011). Despite *B. bacilliformis* and *B. quintana*, for which humans serve as reservoirs, all *Bartonella* spp. linked to human disease originate from wildlife. Several arthropod species are suspected of transmitting bartonellae with cycles in wildlife and livestock, predominantly ticks and fleas (Billeter et al., 2008; Tsai et al., 2011; Vayssier-Taussat et al., 2009). Yet, vectorial ability has only been conclusively identified for the arthropods transmitting the three most important human diseases, *Lutzomyia verrucarum* sandflies (*B. bacilliformis*), *Ctenocephalis felis* fleas (*B. henselae*), and *Pediculus humanus humanus* body lice (*B. quintana*), besides for *Ctenophthalmus nobilis* fleas transmitting bartonellae to rodents (Bown et al., 2004).

Members of this genus persist in endothelia cells and can establish a long lasting intra-erythrocytic bacteremia (Chomel et al., 2009). For its hemolytic capability *B. bacilliformis* induces pronounced anemia in the course of Oroya fever, which explains the severity of the disease. Cardiac infections and organ failures can occur and lead to up to 90% mortality in untreated persons (Maguiña and Ordaya, 2013). *Bartonella henselae* infection shows a comparatively mild course of disease and good cure without special treatment at least in immune competent persons. The common mode of transmission are flea feces that are inoculated by cats that scratch humans with contaminated claws. The disease is therefore called cat scratch disease. Recurrent high fever episodes with headaches lasting five days each are characteristic for trench fever (Kempf and Autenrieth, 2016). *Bartonella quintana* multiplies in the louse's intestine and the disease is transmitted by inoculation of *B. quintana* infected feces from lice to humans. The transmission to lice is facilitated by feeding on bacteremic humans (Foucault et al., 2006). Ruminants have shown to be infected by *B. bovis*, *B. capreoli*, *B. chomelii*, *B. melophagi* and *B. schoenbuchensis* with varying severity of disease (Chomel et al., 2009; Maillard et al., 2007; Vayssier-Taussat et al., 2009).

Yersinia pestis is a species of gamma-proteobacteria in the order of Enterobacteriales. It is particularly notable as the causative agent of plague and for being responsible for three historical pandemics (Prentice and Rahalison, 2007). Today there are several persisting plague foci worldwide with Madagascar accounting for the most annually reported cases and deaths (Bertherat, 2016; WHO, 2017). Rodents appear as mammal reservoir hosts, and fleas as prime vectors. *Yersinia pestis* can also be transmitted directly via contact to infected animal body fluids.

In mammals, infection with *Y. pestis* ranges from an asymptomatic course of disease to high mortality in some species (Hurst, 2018). Bubonic plague follows an infection by flea bites, showing characteristically swollen lymph nodes. A septemic plague can develop in the further progress, and bacteria entering the lung by hematogenous spread cause the highly progressive and infective pneumonic form of the disease. Pneumonic plague is human-human transmitted by aerosols and shows high lethality (Gage and Kosoy, 2005). *Yersinia pestis* is able to persist in soil for months and infect burrowing rodents, which is the suspected main reason for the long term persistence of plague foci in nature (Ayyadurai et al., 2008; Gage and Kosoy, 2005). More than a hundred flea species are vectors among 200 species of rodents. The rat flea *X. cheopis* is the most important vector for human plague, but the human flea (*Pulex irritans*) and the body louse have also been suggested (Ayyadurai et al., 2010; Laudisoit et al., 2007).

Objectives

Madagascar is not only rated as a hotspot in terms of biodiversity but also as an emerging disease hotspot (Brito et al., 2012; Jones et al., 2008). With the majority of emerging human diseases being of zoonotic origin (Taylor et al., 2001), bacteria account for more than the half of emerging infectious disease (EID) events and estimated 22.8% EIDs are transmitted by vectors (Jones et al., 2008). Factors promoting disease transmission are well known for Madagascar, the presence of ectoparasites and potential pathogens, however, is sparsely discovered. At the same time Madagascar reveals a poorly developed public health system, and especially in rural areas there is a lack of human and animal health facilities (Barmania, 2015), and vector-borne infections may occur but remain unseen.

Chapter 2 aims on investigating the diversity of ectoparasites which are hosted by the mammal community in Tsimanampetsotsa, considering invasive rodents and livestock, two important threats of the local ecosystem in order to bring light to ectoparasites they may carry into the ecosystem. The influence of seasonality and habitat degradation on ectoparasite burdens are also addressed.

Chapter 3 is dedicated to bacteria that are commonly detected in ectoparasites, but poorly studied in Madagascar: The genera *Rickettsia*, *Borrelia* and *Bartonella*, all of which have members pathogenic to both animals and humans. In contrast, *Y. pestis* is well known to cause numerous infections and deaths every year, but principally in central Madagascar, and is therefore included for regional surveillance. The immanent zoonotic potential provides a reasonable access to study the occurrence of the mentioned pathogenic bacteria, as required for estimating regional disease risk.

This thesis investigates the following questions:

- Which ectoparasites are present in the study area?
- Are there ectoparasite species which are shared between different host species?
- Is there a contribution of introduced species to the parasite burdens of endemic mammals?
- Is parasite prevalence correlated with habitat disturbance or season?
- Which pathogens are harbored by the examined vectors in question?
- Is there a risk for (cross-species) disease transmission of vector-borne pathogens?

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Chapter 2

Ectoparasites of endemic and domestic animals in southwest Madagascar

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Abstract

Human encroachment of natural habitats bears the threat of disease transmission between native and introduced species that had not come into contact before, thus promoting the spread of new diseases in both directions. This is a matter of concern especially in areas where human-wildlife contact has not been intense in the recent past. In southwest Madagascar, we collected ectoparasites from various mammalian hosts and chicken, and examined their host preferences and their prevalence in relation to season and habitat degradation.

Field-work took place in the northern portion of Tsimanampetsotsa National Park and the adjacent coastal strip (littoral) in the dry and in the rainy season of 2016/2017. Endemic mammals were trapped with live traps placed in habitats of different degrees of degradation: 1) relatively pristine forest, 2) degraded forest, 3) cultivated and shrub land. Rats and mice were also trapped in 4) villages.

We identified 17 species of ectoparasites (296 individuals of ticks [5 species], 535 lice [7 spp.], 389 fleas [4 spp.] and 13 mites [1 sp.]) collected from 15 host species. There was no indication for seasonal or habitat effects on parasite infection. A large portion of the parasites was host-specific. Some ectoparasite species were shared either by several endemic or by several introduced species, but apart from the introduced flea species *Echidnophaga gallinacea* (collected from six different hosts including the endemic carnivore *Galidictis grandidieri*) no other ectoparasite species was shared between endemic and introduced host species.

Introduction

Driven by a rapidly growing human population (UNFPA, 2019), the destruction of forest habitats is a major concern in Madagascar (Miles et al., 2006; Harper et al., 2007, Ratovonamana et al., 2013; Brinkmann et al., 2014). The anthropogenic alteration of the environment and the increase in contact zones between anthropogenic and original habitats represents a potential threat for humans and wildlife alike, as new contacts are important drivers of the emergence of new diseases and their transmission between livestock, humans and wildlife (Daszak et al., 2000; Dobson and Foufopoulos, 2001; Köndgen et al., 2008; Gortazar et al., 2014; Plowright et al., 2015).

In Madagascar, studies on wildlife pathogens have focused on viral or bacterial disease transmission or intestinal endoparasites (Raharivololona et al., 2007; Irwin and Raharison, 2009; Wright et al., 2009; Schwitzer et al., 2010; Bublitz et al., 2015; Radespiel et al., 2015; Zohdy et al., 2015; Rakotoniaina et al., 2016). Studies on ectoparasites are scant (reviewed by Klompen, 2003; O'Connor, 2003), and studies on lemur parasites (Junge and Louis, 2007; Dutton et al., 2008; Junge et al., 2008; Durden et al., 2010; Rodriguez et al., 2012; Klein et al., 2018) outnumber those of ectoparasites from non-lemur taxa, such as tenrecs (Apanaskevich et al. 2013) and rats (Brook et al. 2017) in recent years. Interspecific interactions between parasites and hosts and the underlying dynamics remain unclear.

Ectoparasites can have a direct impact on the health of the infested animal by causing blood loss or skin lesions and inducing inflammatory responses (Brain and Bohrmann, 1992). Some tick species secrete toxins while feeding that may cause tick paralysis in the host, which is likely the severest of these direct reactions to ectoparasitic infestation, often with fatal outcome (Masina and Broady, 1999). Indeed, dozens of Verreaux's sifakas (Propithecus verreauxi) infested with large numbers of ticks died in southern Madagascar in 2018 possibly through tick paralysis as indicated by paralyzed limbs prior to apnea (Bittel, 2018; Carver, 2018).

Above all, ectoparasites are of interest as vectors of pathogens (Reeves et al., 2006; de la Fuente et al., 2008; Rakotonanahary et al., 2017). The black rat (Rattus rattus) acts as a host for various ectoparasites (Duplantier and Duchemin, 2003b; Klompen, 2003), and serves as reservoir of a variety of vector-borne pathogens such as Yersinia pestis (Duplantier and Duchemin, 2003a). This is of relevance as black rats are not only associated with humans but also enter natural habitats (Goodman, 1995; Ganzhorn, 2003; Scott et al., 2006). In addition, as a part of the traditional livestock husbandry, cattle are driven over large distances in search for food and water in many parts of Madagascar (Ratovonamana et al., 2013; Feldt and

Schlecht, 2016; Goetter, 2016), thereby crossing both degraded and protected areas. The increased contact with the endemic fauna can result in pathogen transmissions between human settlements, cultivated land and pristine habitats.

Apart from the increase in contact zones between humans and wildlife, the degradation of pristine habitat per se is assumed to increase parasite loads and susceptibility to diseases in native wildlife, though this may not always be the case (Civitello et al. 2015; Rakotoniaina et al. 2016).

Here, we examine ectoparasites of livestock and endemic Malagasy vertebrates along a gradient of land use ranging from natural forest to agricultural land and villages in the dry south-western part of Madagascar. Specific questions were: (1) Which ectoparasites are present on different hosts in the area, and which are shared between them, thus providing the chance for the transmission of vector-borne diseases? (2) Does parasite prevalence correlate with habitat disturbance, and does it show seasonal variation? (3) Does transhumance contribute to the spread of ectoparasites?

Methods

Study site and period

The study was conducted from June to August 2016 (dry season) and from October 2016 to April 2017 (wet season) in south-western Madagascar (Betioky district, Atsimo-Andrefana Region, south of Tulear), at the western edge of the northern part of Tsimanampetsotsa National Park (TNP) and between TNP and the coast (Figure 2.1). The region is characterized by irregular rainfall of about 300-350 mm per year and an annual mean temperature of 24°C (Ratovonamana et al., 2011). The plateau to the east of the coastal zone receives more rain and provides fodder for livestock during the wet season.

Within the spiny forest ecosystem (Moat and Smith, 2007), the degradation categories follow Steffens et al. (2017) and range from intact dry spiny forest in TNP (class 1) to an open anthropogenically altered landscape of cultivated fields with hedges bordering on sparsely vegetated shrubland at the coast (class 3). In between, there are forest fragments and dry spiny forest degraded by wood collection and livestock grazing (class 2). Villages were assigned to class 4.

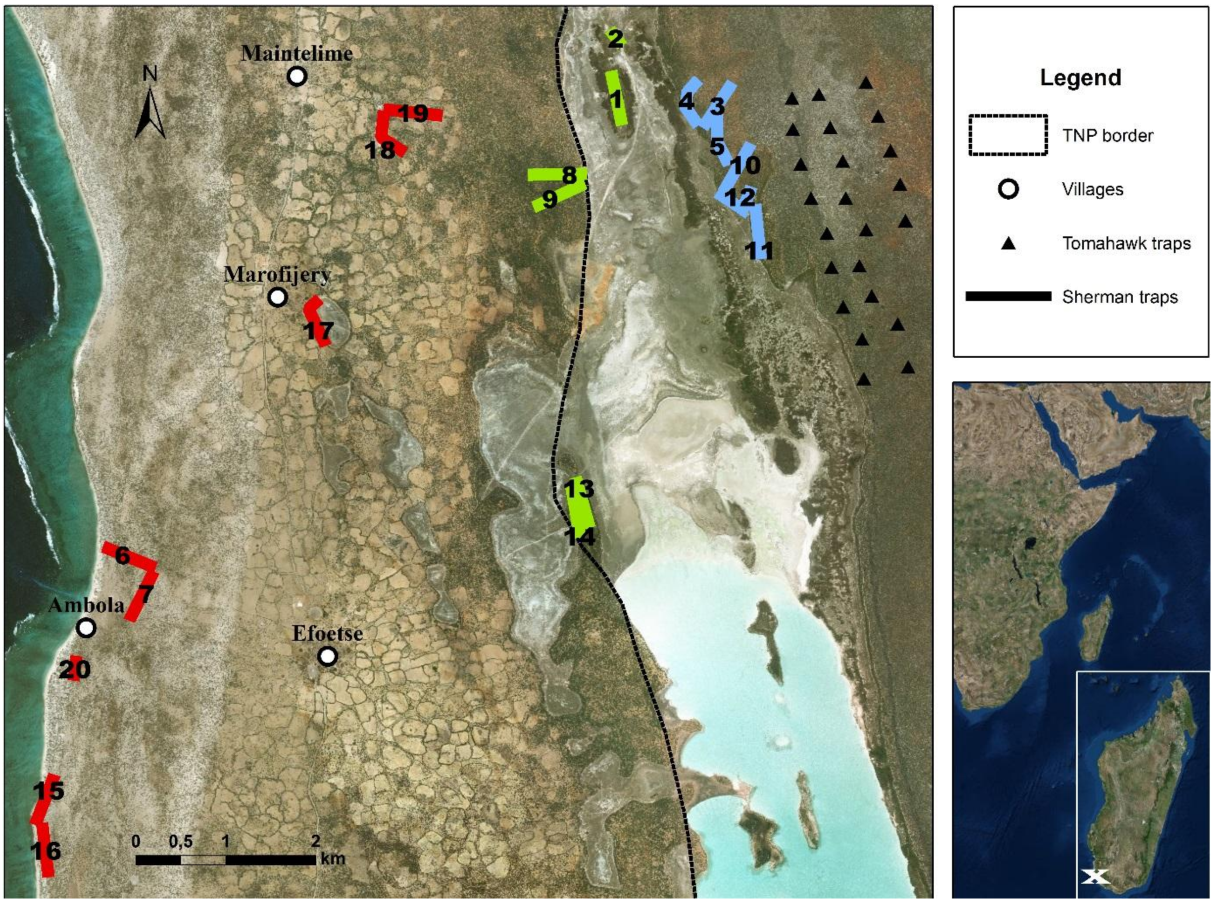

Figure 2.1. Map of the study area (littoral) showing locations of Sherman traps line transects (for small mammals, the color indicates the class of habitat degradation: blue = class 1, green = class 2 and red = class 3) and tomahawk traps (for *G. grandidieri*) in a grid (Geodetic system: WGS 84, map source: Esri, DigitalGlobe, GeoEye, Earthstar Geographics, CNES/Airbus DS, USDA, USGS, AeroGRID, IGN, and the GIS User Community).

Sampling of small mammals, lemurs and carnivores

Small mammal including the lemur species *Microcebus griseorufus* were trapped on 20 transects with Sherman live traps (7.7 x 7.7 x 23 cm) along the gradient of degradation (Figure 2.1) following standard procedures (Rakotondranary et al., 2010; Bohr et al., 2011). Six transects were installed in habitat classes 1 and 2 each, and another eight transects were set within habitat class 3. Each transect comprised 20 trapping points at 25 m intervals. Two traps were installed at every point: one on the ground and the other one placed in trees or shrubs at a height of 1 - 2 m. Trapping was carried out for four consecutive nights per transect. Synanthropic rats (*R. rattus*) and mice (*Mus musculus*) were trapped in huts of the villages Efoetse (E 43.6998, S 24.0786) and Marofijery (E 43.6948, S 24.0427) for two consecutive nights with two Sherman traps in each of ten huts. In the afternoon, traps were baited with banana, and controlled after sunrise the next morning.

The larger endemic carnivore *Galidictis grandidieri* (giant-striped mongoose) was trapped in a grid of tomahawk traps baited with goat skin some 0.5 km east of the line transects within TNP (Figure 2.1; for details see Marquardt et al., 2011).

Animals were scanned for ectoparasites using tweezers and a comb. Animals were marked by ear punches to allow identification of recaptures and released at the site of capture. Lemurs were anesthetized by i.m. injection of 0.02 mL ketamine 10% (WDT, Garbsen, Germany) (except for lactating females in the rainy season, which were examined at the point of capture and directly released after examination). Rats and mice were sacrificed before examination by cervical dislocation and sprayed with ethanol to immobilize hosted fleas. Disposable gloves were worn during examination. Between examination of different individuals, gloves were changed and instruments were disinfected using ethanol.

Sampling of livestock, pets, poultry and tortoises

Local herders would not allow systematic sampling of their livestock (i.e. goats, sheep and cattle) by foreigners. Therefore, local assistants collected ectoparasites from these animals opportunistically. In order to assess the effects of transhumance, cattle were also sampled on the plateau, where they are moved to for grazing at the beginning of the wet season in December. They are brought back to the littoral in March/April as the littoral provides water year round while the plateau does not (Ratovonamana et al., 2013). We assume that parasite collections from single hosts are incomplete and therefore did not calculate intensities of parasite infestation for livestock.

Small parasite collections from cats, dogs and poultry were received from opportunistic collections from villages conducted by S. J. Rakotondranary. Data from free-ranging tortoises of the study site were taken from Ehlers et al. (2016).

Parasites collection and identification

Ectoparasites were preserved in 2 mL tubes in absolute ethanol. Specimens (except mites) were examined with an Olympus SZ61stereomicroscope (Olympus Corporation, Shinjuku, Tokyo, Japan) and identified to species based on morphological characteristics (Ward, 1951; Uilenberg et al., 1979; Varma, 1993; Pajot, 2000; Duchemin, 2003; Walker et al., 2014). Mites were mounted after boiling at 122°C in 85 % lactic acid and examined with a Zeiss Axioscope 2 (Carl Zeiss AG, Jena, Germany). Representative specimens of each parasite species were imaged either with integrated cameras (Bresser MicroCam [Bresser Corporation, Rhede, Germany] with Olympus, Nikon Coolpix 950 [Nikon Corporation, Shinagawa, Tokyo, Japan] with Zeiss), or with a custom-made BK Plus Lab System by Dun, Inc. (Palmyra, Virginia, USA) including Canon camera, macro lenses (65mm and 100mm), and the BK stacking software.

Statistics

Statistical tests were conducted with R (RCoreTeam, 2018) using RStudio (Version 1.0.44).

Results

Capture rates and host abundance

Systematic sampling along the 20 transects for small mammals resulted in a total of 6762 trap-nights (Table 2.1). The insectivorous tenrecs *Echinops telfairi* and *Setifer setosus* are obligatory hibernators and were caught only during the hot wet season (Table 2.1). Seven individuals of the endemic rodent *Macrotarsomys bastardi* (big footed mouse) were caught in all vegetation types. For this species and the tenrecs sample sizes were too small for statistical analyses. *Microcebus griseorufus* can go into torpor but capture rates did not differ between the wet and the dry season within transects (Wilcoxon matched-pair-signed-rank test: W = 27, p = 1, N = 15 transects where traps were set in both seasons). For *R. rattus* capture rates tended to be higher during the dry than during the wet season (Wilcoxon test: W = 20, p = 0.058, N = 15 transects). In villages rats and mice were also caught in both seasons (Table 2.1). Apart from the two tenrec species, there was no indication for seasonal differences in the abundance of host species (Table 2.1). Therefore, seasonal variation in host abundance was not considered in the analyses any further.

Table 2.1. Transect information for the systematic sampling of small mammals, including *M. griseorufus*. Numbers are individuals caught without recaptures. Habitat: TNP = Tsimanampetsotsa National Park; deg. For. = degraded forest; Fields = Agricultural fields. Habitat classification ranges from 1 (= intact forest) to 4 (villages). Species abbreviations: Lemurs*: Mg = Microcebus griseorufus;* Rodents: *Rr = Rattus rattus; Mm = Mus musculus; Mb = Macrotarsomys bastardi;* Insectivores: *Et = Echinops telfairi; Ss = Setifer setosus.*

Transect	Habitat	Habitat class	Dry season						Wet season					
			(June – August 2016)						(Oct. – Dec. 2016 and Feb. – April 2017)					
			Mg	*Rr*	*Mm*	*Mb*	*Et/Ss*	Trap nights	*Mg*	*Rr*	*Mm*	*Mb*	*Et/Ss*	Trap nights
T03	TNP	1	3	0	0	0	0	200	0	0	0	0	0	160
T04	TNP	1	4	0	0	3	0	200	3	0	0	0	0	320
T05	TNP	1	6	0	0	0	0	160	12	0	0	1	0	320
T10	TNP	1	1	2	0	0	0	160	1	1	0	0	0	160
T11	TNP	1	4	0	0	0	0	160	2	0	0	0	0	160
T12	TNP	1	3	0	0	0	0	160	1	0	0	0	0	160
Camp	TNP	1	2	4	0	0	0	50	2	2	0	0	0	60
T01	deg. For.	2	8	4	0	0	0	320	17	2	0	0	0	320
T02	deg. For.	2	0	0	0	0	0	160	6	0	0	0	0	160
T08	deg. For.	2	3	0	0	1	0	160	4	0	0	0	2	160
T09	deg. For.	2	0	1	0	0	0	160	6	0	0	0	0	160
T13	deg. For.	2	1	0	0	0	0	160	-	-	-	-	-	-
T14	deg. For.	2	1	0	0	0	0	160	-	-	-	-	-	-
T17	Fields	3	2	1	0	0	0	160	4	0	0	0	0	320
T18	Fields	3	3	0	0	0	0	160	2	0	0	0	0	320
T19	Fields	3	0	0	0	0	0	160	0	1	0	0	0	328
T06	Shrub	3	0	0	0	1	0	160	-	-	-	-	-	-
T07	Shrub	3	0	0	0	0	0	160	-	-	-	-	-	-
T15	Shrub	3	0	0	0	1	0	160	0	0	0	0	0	160
T16	Shrub	3	0	0	0	0	0	160	-	-	-	-	-	-
T20	Shrub	3	1	0	0	0	0	4	0	1	0	0	1	160
Marofijery	Village	4	0	0	3	0	0	40	0	2	7	0	0	180
Efoetse	Village	4	0	5	8	0	0	40	0	4	17	0	0	180

Mammalian ectoparasites

Seventeen different species of ectoparasites were recorded in the study area, infesting a total of 15 host species (Table 2.2, Figure 2.2). These ectoparasites predominantly belong to ticks (Ixodida: Ixodidae and Argasidae), lice (Phthiraptera: Anoplura: Polyplacidae, Linognathidae, Haematopinidae and Hoplopleuridae; Phthiraptera: Ischnocera: Trichodectidae) and fleas (Siphonaptera: Pulicidae). In addition, one species of mesostigmatid mite was found infesting *R. rattus*, which showed morphological characters of the tropical rat mite *Ornithonyssus bacoti*. The five species of ticks were the endemic *Amblyomma chabaudi, Haemaphysalis simplex* and *Argas echinops* and the introduced *Amblyomma variegatum* and *Rhipicephalus microplus*. Lice were represented by seven species. The only endemic louse species was *Lemurpediculus verruculosus*, found on *M. griseorufus*. A single individual of *Hoplopleura pacifica* was encountered on *R. rattus*. The remaining five species (sucking lice: *Haematopinus quadripertusus*, *Linognathus vituli*, *L. africanus*; chewing lice: *Bovicola caprae, B. ovis*) were all associated with livestock. Four species of fleas included the tropical rat flea *Xenopsylla cheopis* and the cosmopolitan *E. gallinacea*, *Ctenocephalides felis* and *Pulex irritans*.

Ectoparasite species shared between host species

Lice showed a very restricted host spectrum. Five species were found on a single host species. *Bovicola ovis* appeared to be the least host-specific louse, infesting 28% of the sheep and 5% of the goats. *Haematopinus quadripertusus*, regularly infesting cattle (=cattle tail louse), was additionally encountered on one goat. One individual of the cattle tick *R. microplus* was also found on a goat, while two individuals of the endemic rodent *M. bastardi* were infested by one larva of *A. chabaudi*. *Haemaphysalis simplex* infested both tenrec species. The only soft tick species, *A. echinops*, was collected from *E. telfairi*.

Fleas revealed the broadest host spectrum headed by *E. gallinacea* (six host species), which was found on cats, dogs, poultry, rats, mice and the endemic carnivore *G. grandidieri*. *Ctenocephalides felis* was removed from cats and dogs, *X. cheopis* from rats and mice. *Pulex irritans*, the “human flea”, was associated only with chicken (Table 2.2).

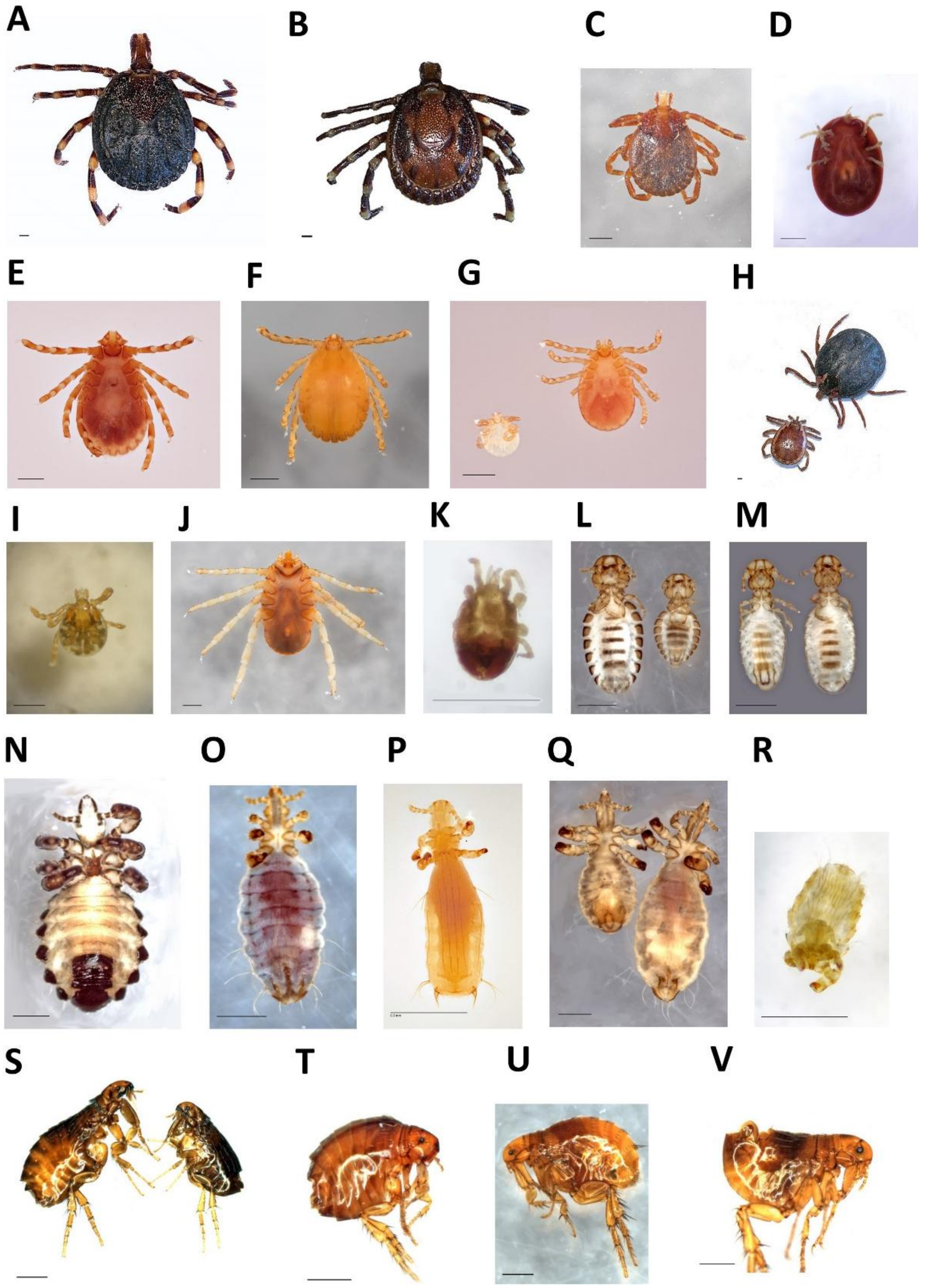

Figure 2.2. Plate of images of the different ectoparasite species. Scale bar = 0.5 mm. A-C *A. variegatum*, dorsal view of female, male and nymph. D *A. echinops* nymph. E-G *H. simplex*, female (ventral), male (dorsal), larva and nymph (ventral). H-I *A. chabaudi*, dorsal view of male, female and larva. J female *R. microplus* (ventral). K *O. bacoti*. L *B. caprae*, ventral view of female and male. M ventral view of *B. ovis* male and female. N *H. quadripertusus* male. O *L. africanus* female. P *L. verruculosus* female. Q *L. vituli* male and female. R *H. pacifica*. S *C. felis* male and female. T *E. gallinacea* female. U *X. cheopis* female. V *P. irritans* male.

Table 2.2. Sampled hosts (rows) and their ectoparasites (columns) in the study area.

		Ticks					Lice							Fleas				Mite
		Amblyomma chabaudi	*Amblyomma variegatum*	*Rhipicephalus microplus*	*Haemaphysalis simplex*	*Argas echinops*	*Lemurpediculus verruculosus*	*Haematopinus quadripertusus*	*Linognathus vituli*	*Linognathus africanus*	*Hoplopleura pacifica*	*Bovicola caprae*	*Bovicola ovis*	*Echidnophaga gallinacea*	*Ctenocephalides felis*	*Xenopsylla cheopis*	*Pulex irritans*	*Ornithonyssus bacoti*
Endemic mammals	*Microcebus griseorufus*						x											
	Macrotarsomys bastardi	x																
	Setifer setosus				x													
	Echinops telfairi				x	x												
	Galidictis grandidieri													x				
Introduced rodents	*Rattus rattus*										x			x		x		x
	Mus musculus													x		x		
Domestic animals and livestock	Cat*													x	x			
	Dog													x	x			
	Goat			x				x		x		x	x					
	Sheep												x					
	Zebu		x	x				x	x									
	Chicken*													x			x	
Tortoises	*Astrochelys radiata***	x																
	*Pyxis arachnoides***	x																

*Samples provided by Rakotondranary (unpubl. data). ** Data from Ehlers et al., 2016.

Table 2.3. Variation of ectoparasite infestation along a degradation gradient in the littoral. Classes of habitat degradation are: (1) relatively pristine forest of Tsimanampetsotsa National Park, (2) degraded forest, (3) cultivated and shrub land, (4) villages (Mean intensities of livestock infestation could not be calculated due to presumed incompleteness of sampling).

	Number of individuals				**Number of infested hosts / prevalence / mean intensity (range)**			
Host species	**habitat class**				**habitat class**			
Parasite species	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Microcebus griseorufus	44	46	12					
Lemurpediculus verruculosus	89	18	8		23 / 52% / 3.9 (1-25)	9 / 20% / 2 (1-4)	5 / 42% / 1.6 (1-3)	
Macrotarsomys bastardi	4	1	2					
Amblyomma chabaudi			2				2 / 100% / 1	
Setifer setosus		1	1					
Haemaphysalis simplex		11				1 / 100% / 11		
Echinops telfairi		1						
Haemaphysalis simplex		8				1 / 100% / 8		
Argas echinops		12				1 / 100% / 12		
Rattus rattus	9	7	3	11				
Hoplopleura pacifica		1				1 / 14% / 1		
Ornithonyssus bacoti	11	1	1		3 / 33% / 3.7 (1-6)	1 / 14% / 1	1 / 33% / 1	
Echidnophaga gallinacea				146				8 / 73% / 18.3 (2-40)
Xenopsylla cheopis				30				8 / 73% / 3.8 (1-6)
Mus musculus				35				
Echidnophaga gallinacea				46				5 / 14% / 9.2 (2-19)
Xenopsylla cheopis				19				9 / 26% / 2.1 (1-4)
Galidictis grandidieri	50							
Echidnophaga gallinacea	64				14 / 28% / 4.6 (1-14)			
Dog				4				
Ctenocephalides felis				14				4 / 100% / 3.5 (1-7)
Echidnophaga gallinacea				20				3 / 75% / 6.7 (6-7)
Goat				151				
Bovicola caprae				78				24 / 16% / NA
Bovicola ovis				15				8 / 5% / NA
Linognathus africanus				54				13 / 9% / NA
Rhipicephalus microplus				1				1 / 1% / NA
Sheep				130				
Bovicola ovis				133				36 / 28% / NA
Cattle				89				
Haematopinus quadripertusus				37				6 / 7% / NA
Linognathus vituli				102				7 / 8% / NA
Amblyomma variegatum				1				1 / 1% / NA
Rhipicephalus microplus				1				1 / 1% / NA

Prevalence of ectoparasites

Habitat disturbance

Except for *M. griseorufus* and *R. rattus*, captures from particular habitats were too low, or restricted to one single habitat (*M. musculus* to villages, *G. grandidieri* to TNP) restraining statistical comparison. In *M. griseorufus*, prevalence of *L. verruculosus* differed significantly between habitats (Fisher's exact $p = 0.005$). The prevalence in habitat class 3 exceeded the prevalence in habitat class 2 (42% vs. 20%) but was highest in class 1 (52%).

Ectoparasite prevalence (all species) of *R. rattus* was three times higher in villages than in the other habitats. Fleas were only present on rats caught inside houses (*E. gallinacea* and *X. cheopis*: 73% prevalence), and mites occurred on rats in all habitats except villages (Table 2.3)

Seasonal variation

Lemurpediculus verruculosus prevalence on *M. griseorufus* was 52% in the dry and 25% in the wet season ($\chi^2 = 6.872$, df = 1, $p = 0.009$, N = 102. On rats, neither fleas nor mites showed different prevalence between seasons (N = 30; fleas: *E. gallinacea*: Fisher's exact $p = 0.70$; *X. cheopis*: $p = 1$; mites: $p = 0.052$), although, all rats infested by mites (N = 5) were caught in the dry season (Table 2.4). Flea prevalence of mice did not differ between the two seasons (*E. gallinacea*: $p = 0.297$; *X. cheopis*: $p = 0.78$). *Galidictis* had a significantly higher prevalence in the wet than in the dry season (83% vs. 11%; $p < 0.001$).

Sex bias

No host species showed sexually biased prevalence (*M. griseorufus*: $\chi^2 = 0.321$, df = 1, $p = 0.57$, N = 83; *R. rattus*: $\chi^2 = 0.031$, df = 1, $p = 0.86$, N = 25; *M. musculus*: Fisher's exact $p = 0.60$, N = 11; *G. grandidieri*: $p = 0.31$, N = 42).

Table 2.4. Seasonal variation of ectoparasite infestation. Mean intensities of livestock infestation could not be calculated (=NA) due to presumed incompleteness of sampling. Opportunistically sampled cats and chicken are excluded.

	Number of individuals		**Number of infested hosts/prevalence/mean intensity (range)**	
Host species	**Season**		**Season**	
Parasite species	**dry**	**wet**	**dry**	**wet**
Microcebus griseorufus	42	60		
Lemurpediculus verruculosus	41	74	22 / 52% / 1.9 (1-3)	15 / 25% / 4.9 (1-25)
Macrotarsomys bastardi	6	1		
Amblyomma chabaudi	2		2 / 33% / 1	
Setifer setosus		2		
Haemaphysalis simplex		11		1 / 50% / 11
Echinops telfairi		1		
Haemaphysalis simplex		8		1 / 100% / 8
Argas echinops		12		1 / 100% / 12
Rattus rattus	17	13		
Hoplopleura pacifica		1		1 / 8% / 1
Ornithonyssus bacoti	13		5 / 29% / 2.6 (1-6)	
Echidnophaga gallinacea	61	85	4 / 23% / 15.2 (6-24)	4 / 31% / 21.3 (2-40)
Xenopsylla cheopis	17	13	5 / 29% / 3.4 (1-6)	3 / 23% / 4.3 (2-6)
Mus musculus	11	24		
Echidnophaga gallinacea	41	5	3 / 27% / 13.7 (8-19)	2 / 8% / 2.5 (2-3)
Xenopsylla cheopis	3	16	2 / 18% / 1.5 (1-2)	7 / 29% / 2.3 (1-4)
Galidictis grandidieri	38	12		
Echidnophaga gallinacea	20	77	4 / 10% / 5 (1-8)	10 / 83% / 7.7(3-14)
Dog		4		
Ctenocephalides felis		14		4 / 100% / 3.5 (1-7)
Echidnophaga gallinacea		20		3 / 75% / 6.7 (6-7)
Goat	23	128		
Bovicola caprae		78		24 / 19% / NA
Bovicola ovis		15		8 / 6% / NA
Linognathus africanus		54		13 / 10% / NA
Rhipicephalus microplus	1		1 / 4% / NA	
Sheep	14	116		
Bovicola ovis		133		36 / 31% / NA
Cattle	18	71		
Haematopinus quadripertusus		37		6 / 8% / NA
Linognathus vituli		102		7 / 10% / NA
Amblyomma variegatum	1		1 / 6% / NA	
Rhipicephalus microplus		1		1 / 1% / NA

Mean intensity of ectoparasite infestation

Habitat disturbance

Since *L. verruculosus* and the rat mite (*O. bacoti*) were the only parasite species present in several habitats, the analysis of habitat disturbance was limited to these two species (Table 2.3). The mean intensity of *L. verruculosus* infestation did not differ between habitats (Kruskal-Wallis: $\chi^2 = 1.924$, df = 2, p = 0.38). The intensity of mite infestation tended to be higher in TNP (3.7 mites per infected host in habitat class 1, and 1 mite per infected host in habitat classes 2 and 3), but the number of mite-infested rats was too low for statistical analyses (N = 5). Mean intensities did not show seasonal variation or any sex bias.

Seasonal prevalence of ectoparasites associated with livestock

Goats, sheep and cattle screened in the vicinity of the trapping transects showed strong seasonal differences especially in the prevalence of lice that were exclusively present in the wet season (Table 2.4). There was no seasonal variation in ticks infesting cattle, given that only one individual of *R. microplus* had been collected in the wet season (Fisher's exact p = 1) and a single *A. variegatum* in the dry season (Fisher's exact p = 0.202).

Transhumance

The 54 cattle sampled in the littoral between August and November 2016 (N = 18 per month) harbored two ticks, while the same number of ticks was found on only nine cattle on the plateau. The louse species *H. quadripertusus* and *L. vituli* were recorded in October and November with a slightly rising prevalence (October: *H. quadripertusus* 11%; *L. vituli*: 17%; November: 22% each). 238 cattle screened on the plateau in March and April 2017, shortly before transhumance to the littoral and 35 cattle screened in the littoral after having returned from the plateau revealed a pronounced difference in tick prevalence (Fisher's exact p = < 0.001, N = 273). *Amblyomma variegatum* was present on 6%, and *R. microplus* on 24% of the cattle grazing on the plateau. The cattle herd in the littoral was tick-free.

Discussion

This study reports ectoparasites on mammals, poultry and tortoises in south-western Madagascar. Screening of 15 endemic and domestic host species revealed 17 different species of ticks, lice, fleas and mites. *Microcebus griseorufus* was the host species caught most frequently by systematic trapping. The sucking louse *L. verruculosus* occurred on 36% of 102 host individuals. Yet, none of these individuals was infested by ticks. The ectoparasites of the small nocturnal lemurs, as *Microcebus* spp., have recently been reviewed by Zohdy and Durden (2016). Studies have found *Microcebus* spp. to host the tick species *Ixodes lemuris*, *Haemaphysalis lemuris* and possibly *H. simplex* (Rodriguez et al., 2012; Blanco et al., 2013). Klein et al. (2018) proposed a third *Haemaphysalis* species named "Candidatus *Haemaphysalis microcebi*". At the Beza Mahafaly Special Reserve (BMSR), some 100 km northeast of our study site, *M. griseorufus* was commonly infested by ticks (74 out of 249 individuals, Rodriguez et al., 2012). In addition, ring-tailed lemurs (*Lemur catta*) are serving as hosts for *H. lemuris* at BMSR (Loudon et al., 2006), but not at TNP (Dutton et al., 2003).

Two species of sucking lice are known to parasitize *Microcebus* spp., both belonging to the genus *Lemurpediculus.* The first described species of this genus was *L. verruculosus*, found on an unspecified "mouse lemur" in the surroundings of Fort Dauphin (Ward, 1951, then *Lemurphtirus verruculosus*). The next record of *L. verruculosus* was from brown mouse lemurs (*Microcebus rufus*) in Ranomafana (Durden et al., 2010). An individual louse identified tentatively as *L. verruculosus* was recovered from *M. griseorufus* at the BMSR (Rodriguez et al., 2012). With our louse collection we now confirm that *M. griseorufus* is another host of this louse species and suggest that *L. verruculosus* is indeed less host specific than thought.

The endemic rodent *M. bastardi* is rarely trapped by our trapping procedure (Ganzhorn, 2003; Youssouf and Rasoazanabary, 2008). It had never been trapped in the study area before during ten years of trapping. Two individuals were infested with *A. chabaudi* larvae while adult *A. chabaudi* are known from the endemic tortoises (Ehlers et al., 2016). Only a few *Haemaphysalis* ticks have been associated with *M. bastardi* in the past (Uilenberg et al., 1979). Here we present the first record of *A. chabaudi* infesting the rodent *M. bastardi*. So far, this tick has been considered as a strictly host specific tortoise tick.

The two tenrec species *S. setosus* and *E. telfairi* are the preferred hosts of *H. simplex,* but Uilenberg et al. (1979) reported this tick also from *Tenrec ecaudatus*, *M. bastardi* and in rats' nests. It had also been recovered from lemurs, the sifaka *Propithecus verreauxi*, probably an accidental host (Uilenberg et al., 1979), and *Microcebus griseorufus*, where it could be more common (Rodriguez et al., 2012). In the TNP region we found *H. simplex* on the two tenrec species, but dissenting from the literature (Hoogstraal and Kim, 1985) we encountered this tick species among the tenrecs' body spurs, not in the ears. *Haemaphysalis simplex* was neither associated with rodents (N = 6 *M. bastardi* and N = 19 *R. rattus*) nor with mouse lemurs.

Rats receive special attention in Madagascar due to the repeated emergence of plague. Two species of fleas are involved in plague circulation: *X. cheopis*, which was introduced together with its host, and the endemic *Synopsyllus fonquerniei*. The latter is known from rats living outside human settlements. This is important for possible sylvatic plague cycles and suspected transmission to endemic rodents with consequent die-off (Duplantier and Duchemin, 2003a, Duplantier et al., 2005). In contrast, *X. cheopis* generally infests rats inside houses (Duplantier and Duchemin, 2003a) as also observed in the present study. Eight out of 11 rats (73%) caught in houses were infested by *X. cheopis*. The same proportion was found hosting a second flea species, the sticktight flea (*E. gallinacea*). We also observed mice serving as hosts for both flea species. The coexistence of mice could be an important factor in maintaining flea populations. *Paractenopsyllus* represents another genus of fleas known to parasite *R. rattus* (Duchemin 2003, Hastriter and Dick, 2009. This genus was reported to be predominantly hosted by shrew tenrecs (*Microgale*) and rodents of the genus *Eliurus* from rain forests of the central highlands or the northern mountains of Madagascar (Duchemin, 2004; Hastriter and Dick, 2009). Consistently, we did not find *Paractenopsyllus* fleas at our study site, where most main hosts are absent (except *Eliurus myoxinus,* Soarimalala and Goodman, 2011) and the climate is much drier.

Apart from fleas, O'Connor et al. (2003) listed five species of mites infesting rats, and seven tick species have been recovered from rats across the country (Uilenberg et al., 1979). It thus seems prudent to suggest that rats are suitable vehicles for transmission of further ectoparasite species between mammalian hosts. Nevertheless, the free-ranging rats trapped at TNP did not reveal any ectoparasites except for mesostigmatid mites, which have not been found on any other host (Table 2.2). Laelapid mites were seen on 17 out of 20 *L. catta* at TNP (Dutton et al., 2003). In BMSR even all *L. catta* individuals had mesostigmatid mites (Loudon et al., 2006). However, due to uncertain identification of mites, it remains unclear if *L. catta* from different regions share the same species of parasitic mite.

Galidictis grandidieri was infested by introduced *E. gallinacea* fleas. No record of ectoparasites has been published or deposited in the Global Mammal Parasite Database (GMPD, www.mammalparasites.org; Nunn and Altizer, 2005). Since *G. grandidieri* occurs from the original forest to the vicinity of human settlements (Marquard et al., 2011), it could easily be infected by fleas when feeding on leftovers of chicken, infested by *E. gallinacea*. *Echidnophaga gallinacea* is not known as a vector for specific diseases but can cause severe skin irritations and a general decline in health. No such signs were noted in *G. grandidieri*.

Habitat disturbance

Habitat disturbance is supposed to correlate positively with ectoparasite prevalence (Wright et al., 2009; Junge et al., 2011) due to nutritional stress and consequently impaired immune functions or due to reduced biodiversity (Beck and Levander, 2000; Chapman et al., 2005; Irwin et al., 2010; Civitello et al., 2015). The infestation of the endemic *G. grandidieri* by an introduced parasite species demonstrates an undeniable anthropogenic effect. Yet, only *M. griseorufus* was caught in sufficient numbers throughout the different habitats to allow statistical comparison. The mean intensity of louse infestation did not differ between the habitats. The prevalence of louse infestation, however, was notably higher in the habitat of degradation class 3 (42%) than in the second most degraded habitat (20%), but it was even higher in TNP (52%). This result contrasts with the assumption that habitat degradation leads to higher ectoparasite prevalence. This finding is inconsistent with the “biodiversity dilution effect” that postulates decreased loads of host-specific parasites with increasing number of species (Civitello et al., 2015). Thus, other (or additional) ecological characters overwrite the possible effects of habitat quality and contacts by livestock and domestic animals. Possible candidates are increased social contacts due to higher population densities in pristine than in degraded habitats (Kappeler et al., 2015; Nunn et al., 2015).

Other impacts

Host density, for example, is crucial for survival of ectoparasite populations. A higher host density would mean a higher possibility for the parasite to find a suitable host (in the case of a tick). And it would also increase the chance of the parasite to actively change the host or to be transmitted from one host to another given that host individuals from dense populations more often encounter conspecifics. It is true that, by offering stable food resources, habitat quality favors population density (Balko and Underwood, 2005), but, on the other hand, this can also be mediated by habitat loss or fragmentation. In a shrinking habitat the density of inhabiting populations would rise, at least initially (Debinski and Holt, 2000). Both could be valid for our case. Nevertheless the density proxy of mouse lemurs we calculated (animal captures per 100 trap nights) rises with habitat quality (from habitat class 3 to 1: 2.5, 3.5, 3.8), which could illustrate the higher prevalence in the pristine forest following a higher density, but giving no explanation for the interrupting decline of prevalence in habitat class 2.

There are also conceivable behavioral aspects influencing prevalence and intensity of infestation with ectoparasites. Grooming is an important social activity in primates to reduce ectoparasite loads (Hutchins and Barash, 1976; Akinyi et al., 2013). Nevertheless, lemurs are not able to use their hands or lips for grooming like higher primates, but use their "tooth comb" to groom themselves or group members (Sauther et al., 2002). In consequence some body parts like the face, head, neck and parts of the upper back are inaccessible for autogrooming lemurs (Hutchins and Barash, 1976), so that they depend on allogrooming in social relationships to clean these regions from ectoparasites. This situation means a disadvantage for solitary individuals.

Recent studies have also shown that sleeping ecology may have an impact on parasite infestation (Hokan et al., 2017; Klein et al., 2018). Hence, sleeping in tree holes apparently increases infestation with mites, lice and ticks, and sleeping in groups promotes the infestation with lice. While these studies and our study on pair-living or solitary species found independency of ectoparasite prevalence from host gender, males of the multi-male multi-female group living *Lemur catta* in BMSR hosted more mites (Sauther et al., 2006) and more ticks and lice than females (Sauther et al., 2002).

Seasonal effects

Ectoparasite prevalence and intensity are assumed to be higher during the wet season, when environmental conditions are favorable for parasite survival (Altizer et al., 2006). The louse infestation of the sheep and goats in the littoral of our study site matches this assumption. Yet, we also observed a strong - albeit reverse - seasonal pattern in louse infestation of *M. griseorufus*. Half of the lemurs trapped in the dry season was infested by *L. verruculosus*, compared to only a quarter of individuals being infested in the wet season. In Ankarafantsika National Park louse prevalence of mouse lemurs was also higher in the dry season, with a peak at its end in October (up to 90%, Klein et al., 2018). However, the prevalence was very variable between years with 3% in May 2015 at the beginning of the dry season, and 74% in May 2016. Other regions or hosts showed a seasonal peak of louse infestation towards the wet season (Wright et al., 2009; Hokan et al., 2017).

At BMSR immature stages of *H. lemuris* have been found on *M. griseorufus* only in the dry season (Rodriguez et al., 2015), whereas adult ticks infest sifakas and ring-tailed lemurs year-round (Loudon et al., 2006). As humidity is claimed to be the most limiting factor for tick survival because of their susceptibility to desiccation (Needham and Teel, 1991; Gray et al., 2009), ticks might reach their distribution limit between the Mahafaly Plateau and the littoral due to decreasing rainfall and humidity.

Ectoparasites of livestock

The six ectoparasite species encountered on livestock are well known in Africa and other tropical regions (Durden and Musser, 1994; Walker et al., 2014), indicating that they have been introduced to Madagascar in the course of livestock trade (Uilenberg, 1964). It is surprising that merely such a small number of livestock-associated ectoparasites had managed to settle in Madagascar. From the some 20 species of ixodid ticks infesting livestock under similar climatic conditions in southern and eastern Africa (Walker et al., 2014), only *A. variegatum* and *R. microplus* have spread to the island. The latter is supposed to have its origin in Southeast Asia, while *A. variegatum* originates from east Africa (Barré and Uilenberg, 2010).

Occurrence of ticks on livestock is affected by seasonality and strongly depends on the tick’s life stage (Yawa et al., 2018). A study from Gambia reports that adult *A. variegatum* infest cattle in the rainy season, and almost disappear in the dry season (Mattioli et al., 1997). In Africa this species occupies all climatic zones, and is supposed to be limited only by arid conditions, i.e. below 400 mm annual rainfall, like in the Sahel (Barré and Uilenberg, 2010). The virtual absence of ticks in the littoral of our study site may also be due to aridity. This observation is consistent with reports by local stockbreeders who claim that cattle are in better health condition in the littoral compared to the plateau.

Lice of livestock were exclusively present in the hot rainy season - a special case, as it is known that louse survival and development is limited by temperature (Matthysse, 1944) and that louse populations of livestock decline in the hottest months in tropical regions and peak in the cold, dry season (Gabaj et al., 1993; Kumar et al., 1994; Rashmi and Saxena, 2017). However, we cannot exclude that our observation is biased by insufficient sampling in the dry season.

Conclusion

This study provides confirmation of a broad spectrum of ectoparasites being present in the examined area. Most ectoparasites found in this study were rather host-specific providing little potential for interspecific disease transmission. Yet, black rats, cats and dogs can transport E. gallinacea fleas into the forest where they can infect endemic carnivores and vice versa. Seasonal or habitat effects were tested significant for louse infestation of M. griseorufus. In contrast to most other studies, the prevalence was highest in the least degraded habitat and showed an increase towards the dry season. Yet, as no further predictors have been recorded, we only can discuss the reasons. We demonstrated that transhumance of cattle towards the drier littoral possibly diminishes tick burdens of a herd.

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Author contribution

I hereby confirm that Julian Ehlers designed the study, conducted sampling in the field and identification of ectoparasites. He analyzed the data and wrote the manuscript.

Jörg Ganzhorn

Prof. Dr. Jörg Ganzhorn

Chapter 3

Molecular detection of *Rickettsia* spp., *Borrelia* spp., *Bartonella* spp. and *Yersinia pestis* in ectoparasites of endemic and domestic animals in southwest Madagascar

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Abstract

Little is known about the presence of vector-borne bacteria in southwest Madagascar. Anthropogenic alteration of natural habitats represents an important driver for the emergence of new diseases. Especially the involvement of livestock and the involuntary maintaining of invasive synanthropic animals (particularly rats) facilitate disease transmission from wildlife to humans and associated animals and vice versa. The dissemination or acquisition of ectoparasites is most likely in regions where human/wildlife contact is increasing. Little is known about the presence of vector-borne bacteria in southwest Madagascar. In 2016 and 2017, ectoparasites were collected from various introduced (cattle and goats, cats, dogs and chicken, rats and mice) and native animal species (mouse lemurs [*Microcebus griseorufus*], Grandidier's mongooses [*Galidictis grandidieri*], bastard big-footed mice [*Macrotarsomys bastardi*], greater hedgehog tenrecs [*Setifer setosus*] and lesser hedgehog tenrecs [*Echinops telfairi*]) in the northern portion of Tsimanampetsotsa National Park and the adjacent littoral region. Thirteen species of blood-feeding ectoparasites (235 individuals of ticks [5 species], 414 lice [4 spp.] and 389 fleas [4 spp.]) were investigated for the presence and identity of rickettsiae, borreliae, bartonellae and *Yersinia pestis* using PCR techniques. *Rickettsia* spp. were detected in every single ectoparasite species (*Amblyomma variegatum, A. chabaudi, Rhipicephalus microplus, Haemaphysalis simplex, Argas echinops, Ctenocephalides felis, Echidnophaga gallinacea, Pulex irritans, Xenopsylla cheopis, Haematopinus quadripertusus, Linognathus africanus, L. vituli, Lemurpediculus verruculosus*). Lice and ticks were found harboring rickettsiae identified as *Rickettsia africae*, while *Rickettsia felis*-like bacteria were associated with fleas. *Borrelia* spp. were detected in 5% of *H. simplex* and 1% of *R. microplus* ticks. *Bartonella* spp. were detected in 40% of *H. quadripertusus* pools and in 5% of *L. verruculosus* pools. *Yersinia pestis* was detected in *X. cheopis* and *E. gallinacea* fleas collected from a rat. This study presents the detection of a broad spectrum of vector-borne bacteria including potential pathogens, and an unexpected finding of *Y. pestis* far off the known plague foci in Madagascar.

Introduction

Approximately 75% of human emerging infectious diseases originate from wildlife and/or domestic animals (Taylor et al., 2001), many of which require ectoparasites for transmission (The World Health Organization (WHO), 2017a). The drivers of emerging infectious diseases have been known for years and are debated intensively (Daszak et al., 2000, Githeko et al., 2000; Patz et al., 2000, 2003; Young et al., 2016). Several factors supporting disease emergence or zoonotic transmission pathways congregate in Madagascar: Importation of domestic animals and introduction of invasive species (Goodman, 1995; Stachurski et al., 2013), alteration of natural habitats through deforestation, replacement of forests with agricultural land, or habitat fragmentation (Harper et al., 2007; Brinkmann et al., 2014). In addition, climate change is expected to alter the distribution ranges of parasite species (Barrett et al., 2013). Biodiversity loss may weaken the dilution effect and hence may trigger increasing parasitism (Dobson et al., 2006; Civitello et al., 2015). Due to the rapid human encroachment of forest habitats the contact zones between synanthropic animals (i.e. livestock [Ratovonamana et al., 2013] as well as rats [Goodman, 1995; Ganzhorn, 2003]) and the endemic wildlife expand. The increased mutual contact can result in extended host spectra for ectoparasites or development of new reservoirs for pathogens in both ways.

The combination with a poor public health infrastructure causes a complex situation in Madagascar. The lack of human and animal health facilities, especially in rural areas, restricts the feasibility of proper diagnosis and treatment as well as the availability of epidemiological data on vector-borne infections (Barmania, 2015). Thus, there is a need for knowing the potential of ectoparasites to transmit diseases and how to prevent it (Bardsley and Thrusfield, 2014). Only a small number of studies about ectoparasitic fauna from recent years present data on harbored pathogens (e.g. Rakotonanahary et al., 2017; Qurollo et al., 2018). Since Madagascar seems to be susceptible to emerging vector-borne diseases to a high degree, there is a demand for investigations of this kind.

Here, we investigated several arthropod vectors (ticks, lice, fleas) from livestock (cattle and goats) and domestic animals (cats, dogs and chicken), peridomestic rodents (rats and mice) as well as endemic small mammals (mouse lemurs (*Microcebus griseorufus*), Grandidier's mongooses (*Galidictis grandidieri*), bastard big-footed mice (*Macrotarsomys bastardi*), greater (*Setifer setosus*) and lesser hedgehog tenrecs (*Echinops telfairi*) for the presence of rickettsiae, borreliae, bartonellae and *Yersinia pestis*. With the present data we provide a first overview of bacterial pathogens circulating in various groups of bloodsucking ectoparasites known for their vector potential, in order to estimate the relevance of vector-borne diseases in southwest Madagascar.

Methods

Study site

The study area is located in southwest Madagascar (Figure 3.1) enclosing the coastal plain at the western edge of Tsimanampetsotsa National Park and parts of the Mahafaly plateau (~200 m a.s.l.) in the east of it. The region belongs to the spiny forest ecosystem characterized by dry and drought adapted vegetation (Fenn, 2003). Its climatic conditions are distinguished by irregular rainfall, often less than 400 mm averaged per year, and an annual mean temperature of 24°C (Ratovonamana et al., 2011). The local population predominantly relies on pastoralism and agriculture. In the surroundings of the villages there are cultivated fields with hedges bordering on sparsely vegetated shrubland.

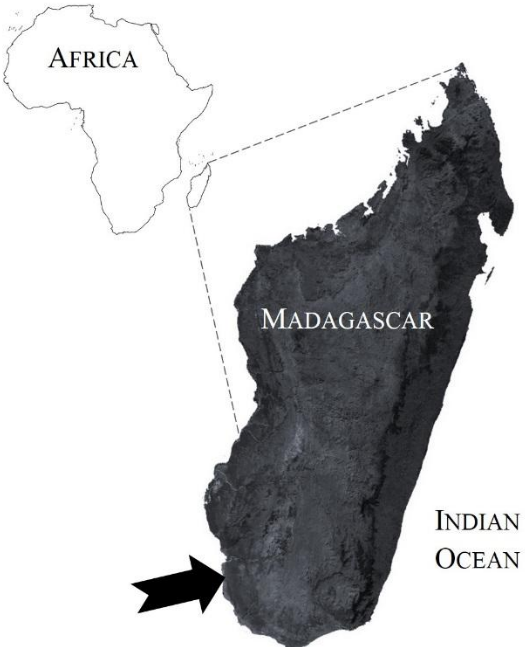

Figure 3.1: Map of Madagascar. Madagascar is located in the Western Indian Ocean close to the coast of East Africa. The arrow points to the study site (E 43.6998, S 24.0786).

Arthropod collection

The arthropods processed in this study, were collected during a project investigating the ectoparasite burdens of mammalian hosts (Ehlers et al., 2019). In brief, ectoparasites were collected from livestock and domestic animals as well as trapped mice, rats and endemic small mammals in the dry season of 2016 and the rainy season of 2017. A smaller portion of the collection was included from sampling efforts conducted at the study site earlier (2013/2014). For a more detailed description of the trapping procedure and arthropod collection see Ehlers et al. (2019). All specimens were stored in 100% ethanol until further processing in the laboratory in Hamburg. The total collection consisted of 13 different ectoparasite species, comprising 1038 individuals (235 ticks, 389 fleas and 414 lice), found on 12 host species. An overview of the mammalian hosts portioned according to their ectoparasites and the results of the bacterial screening is provided in the supplementary Figure S3.

Dissection of rat and mouse organs

Rats and mice were sacrificed by cervical dislocation and sprayed with ethanol to immobilize hosted fleas. Spleens and kidneys were dissected using disposable scalpel blades and stored in 100% ethanol. Personal protective equipment concluded FTP3 dust mask, goggles and disposable gloves.

DNA extraction

DNA of fleas and lice was extracted from pooled individuals (up to ten) resulting in 76 pools of fleas and 102 pools of lice. Ticks were processed individually. Arthropods were dried on filter paper, and transferred to 2.0 mL tubes containing two 3 mm steel beads. Ticks and the solid *Heamatopinus* lice were roughly cut into pieces using pointed scalpel blades (Bayha, Tuttlingen, Germany). The tubes were heated to 50°C for 5 min to evaporate remaining ethanol. The samples were then homogenized in 180 µL PBS using the TissueLyzer LT (Qiagen, Hilden, Germany) for 4 min at 30 Hz. The next steps followed the insect protocol for the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) with minor modifications: The lysing temperature was raised to 65°C and DNA was eluted with 100 µL buffer AE. A negative extraction control was processed along with each batch of arthropods (24 samples). Organ samples were treated according to the standard tissue protocol.

Pathogen DNA detection

Rickettsial DNA was detected by targeting the *ompB* gene with the primers 120-M59 and 120-807 (Roux and Raoult, 2000). Detection of *Borrelia* DNA was performed by combining an initial 16S rDNA screening qPCR (Parola et al., 2011) with conventional PCRs targeting *gyrB* (with three different primer pairs: gyrB-5'+1/gyrB-3'+1 (Schwan et al., 2005), gyrB-F/gyrB-R and gyrB-343/gyrB-1480, both designed for this study), *flaB* (with primers flaB-BOR1/flaB-BOR2 (Assous et al., 2006)), and a larger fragment portion of the 16S rDNA (Barbour et al., 1996). The qPCR reaction proceeded in a 20 µL volume containing 16 µL master mix (0.16 µL Platinum Taq polymerase, 4 mM $MgCl_2$, 0.2 mM each dNTP, 0.6 µM each primer, 0.2 µM probe and 0.04 µM BSA) and 4 µL template DNA. Flea samples were excluded from the *Borrelia* screening.

The presence of *Bartonella* DNA was verified by an initial qPCR targeting the 16S-23S ribosomal RNA intergenic spacer using primers ITS-Bart-F and ITS-Bart-R and the probe ITS-Bart-probe designed in our laboratory. The reaction mix consisted of 10 µL QuantiTect Probe PCR Master Mix, 0.4 µM each primer, 0.2 µM probe and 2µL template DNA in a final volume of 20 µL. Positive samples were confirmed by two consecutive conventional PCRs of the genes *gltA* (Norman et al., 1995) and *rpoB* (Renesto et al., 2001). Tick samples, except *Amblyomma variegatum*, were excluded from the *Bartonella* screening.

Flea DNA samples were additionally screened for *Y. pestis* by qPCR targeting the PCP1 gene using primers YpplaS1 and YpplaAs1 and probe YpplaP (Panning, unpublished). The PCR was performed using HotStarTaq *Plus* DNA polymerase in a 20 µL reaction volume with 0.12 µL of the enzyme, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM each primer, 0.2 µM probe and 2 µL template DNA.

For the conventional PCR assays we used either Platinum Taq DNA polymerase or HotStarTaq DNA polymerase (for *Bartonella gltA* and *Borrelia gyrB*) in a 25 µL reaction volume composed of 0.15 µL polymerase, 1.5 mM $MgCl_2$ (2 mM for HotStarTaq) 0.2 mM each dNTP, 0.4 µM (0.6 µM) each primer and 2 µL template DNA. (Detailed PCR conditions and oligonucleotide sequences are listed in the supplementary Table S2).

Conventional PCRs were run with a Peqlab Primus 96 advanced® thermal cycler (PEQLAB Biotechnologie GMBH, Erlangen, Germany), for qPCR the LightCycler® 480 (Roche Diagnostics GmbH, Mannheim, Germany) was used. A positive and a negative control were included in each run.

Calculation of prevalence and minimal infection rate (MIR)

The prevalence (percentage of infected individuals) was calculated by dividing the number of infected individuals by the total number of tested specimens. The MIR was calculated by dividing the number of positive pools by the total number of individuals from all pools per species.

Sequence analysis

The success of the conventional PCR amplification was verified by electrophoresis of the PCR products on 1% agarose gels in Tris-acetate-EDTA buffer (TAE) and staining with GelRed^{TM} Nucleic Acid Gel Stain (Biotium, Hayward, USA) added to the liquid gel. Positive samples were purified according to the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sent to Microsynth Seqlab (Göttingen, Germany) for sequencing.

DNA sequences were checked for quality and ambiguous signals were trimmed using BioEdit Sequence Alignment Editor (version 7.2.5; Hall, 1999). Basic Local Alignment Search Tool (BLAST) was used for comparison to sequences deposited in the NCBI GenBank (Zhang et al., 2000). Sequences of PCR products and those obtained from GenBank were aligned by using ClustalW (Thompson et al., 1994) implemented in BioEdit. The MEGA 7 software (version 7.0.20; Kumar et al., 2016) was used for phylogenetic analysis by applying the neighbor-joining method (Saitou and Nei, 1987) in a complete deletion procedure, performing 1.000 bootstrap replications (Felsenstein, 1985). The evolutionary distances of the trees were computed using the Kimura 2-parameter method (Kimura, 1980).

Results

Rickettsia screening

OmpB rickettsial DNA was detected in all of the 13 ectoparasite species. In ticks, there was an overall prevalence of 26% (61/235) led by *Amblyomma* ticks (*A. variegatum* and *A. chabaudi*), which revealed a prevalence of 100% (30/30), followed by 16% *Rickettsia* prevalence in *Rhipicephalus microplus* (28/176), 11% in *Haemaphysalis simplex* (2/17) and 10% in *Argas echinops* (1/10; Table 3.1). Regarding fleas, 36 of 76 pools proved to contain rickettsial DNA (MIR 9%). In detail, the partial *ompB* gene was amplified from 5/6 *Ctenocephalides felis* pools (MIR 16%), from 22/49 *Echidnophaga gallinacea* pools (MIR 8%), from 1/4 *Pulex irritans* pools and from 8/17 *Xenopsylla cheopis* pools (MIR 14%; Table 3.2). *OmpB* amplification of lice pools revealed similar infection rates to fleas (overall MIR 10%; 43/102 positive pools). 12/35 *Haematopinus quadripertusus* (MIR 7%), 16/37

Lemurpediculus verruculosus (MIR 17%), 10/22 *Linognathus africanus* (MIR 11%) and 5/8 *Linognathus vituli* pools (MIR 8%) were successfully amplified (Table 3.3).

Table 3.1. PCR based screening results of pathogen DNA isolated from individual ticks (PREV = prevalence; neg = no DNA was detected; pos = positive screening result, genus affiliation confirmed by sequence analysis; n.d. = not done).

	***Rickettsia* sp.**			***Borrelia* sp.**			***Bartonella* sp.**		
Tick species	neg	pos	PREV	neg	pos	PREV	neg	pos	PREV
Amblyomma variegatum (n=28)	0	28	100%	28	0	0%	28	0	0%
Amblyomma chabaudi (n=2)	0	2	100%	2	0	0%		n.d.	
Argas echinops (n=10)	9	1	10%	10	0	0%		n.d.	
Haemaphysalis simplex (n=19)	17	2	11%	18	1	5%		n.d.	
Rhipicephalus microplus (n=176)	148	28	16%	174	2	1%		n.d.	
total (n=235)	174	61	26%	232	3	1%			

Table 3.2. PCR based screening results of pathogen DNA isolated from pooled fleas (MIR = minimal infection rate; neg = no DNA was detected; pos = positive screening result, genus affiliation confirmed by sequence analysis).

	***Rickettsia* sp.**			***Bartonella* sp.**			***Yersinia* sp.**		
Flea species	neg	pos	MIR	neg	pos	MIR	neg	pos	MIR
Ctenocephalides felis (n=31)	1	5	16%	6	0	0%	6	0	0%
Echidnophaga gallinacea (n=289)	27	22	8%	49	0	0%	46	3	1%
Pulex irritans (n=10)	3	1	10%	4	0	0%	4	0	0%
Xenopsylla cheopis (n=59)	9	8	14%	17	0	0%	16	1	2%
total (n=389)	40	36	9%	76	0	0%	72	4	1%

Table 3.3. PCR based screening results of pathogen DNA isolated from pooled lice (MIR = minimal infection rate; neg = no DNA was detected; pos = positive screening result, genus affiliation confirmed by sequence analysis).

	***Rickettsia* sp.**			***Borrelia* sp.**			***Bartonella* sp.**		
Louse species	neg	pos	MIR	neg	pos	MIR	neg	pos	MIR
Haematopinus quadripertusus (n=167)	23	12	7%	35	0	0%	21	14	8%
Lemurpediculus verruculosus (n=96)	21	16	17%	37	0	0%	35	2	2%
Linognathus africanus (n=87)	12	10	11%	22	0	0%	22	0	0%
Linognathus vituli (n=64)	3	5	8%	8	0	0%	8	0	0%
total (n=414)	59	43	10%	102	0	0%	86	16	4%

Analyses of sequenced partial *ompB* PCR products confirmed most of these sequences (16/20) matching to *Rickettsia africae* (Figure 3.2). The sequences shared 99.9% (1/783 mismatch; GenBank accession numbers MN304852, MN304853, MN304858, MN304859, MN304861, and MN304862; detailed BLAST results are listed in the supplementary Table S1) to 100% sequence identity with *R. africae* strain ESF-5 (CP001612). The aberration, a C→T transition (a change from leucine to proline) at position 182, was seen in each one of the louse-derived sequences (MN304863, MN304864, MN304865 and MN304866), by all flea sequences except *P. irritans* (MN304848, MN304849, MN304850, MN304851 and MN304856) and by the tick *A. chabaudi* (MN304860). The PCR product of the remaining four sequences revealed a 6 bp insertion at position 848 (of the ORF) like *Rickettsia felis* and were clustered accordingly in the phylogenetic tree (Figure 3.2). They all originated from flea samples. The detected DNA of a single *C. felis* pool showed 100% homology (789/789 bp; MN304847) compared to those of *R. felis* (CP000053). The other three sequences (MN304854, MN304855 and MN304857) showed only 96.2% homology to *R. felis*, but were closer related (98.4%) to another recently discovered candidate *Rickettsia sp.* called Candidatus *Rickettsia senegalensis* (KU499848). The flea species providing this deviating sequence were *E. gallinacea*, *X. cheopis* and *P. irritans*.

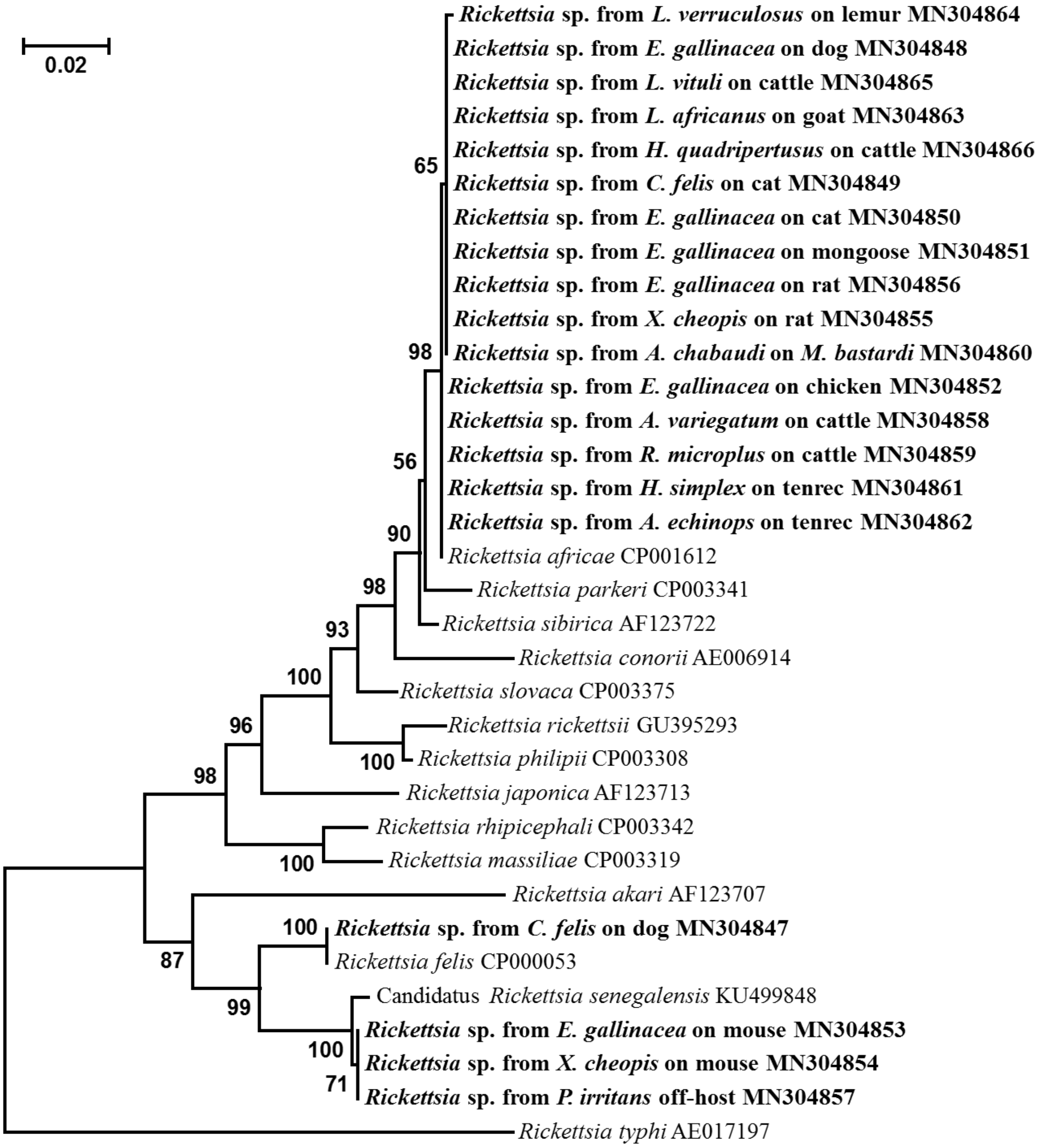

Figure 3.2. Evolutionary relationship of *Rickettsia* spp. based on *ompB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.52448091 is shown. The bootstrap values are shown next to the branches. The tree is drawn to scale. There were a total of 777 positions in the final dataset.

Borrelia screening

The *Borrelia* qPCR generated many false positive results (78/235 tick and 52/110 louse samples were positive), and only three tick samples could be confirmed by at least one of the three consecutive conventional PCRs. Ct-values of false positive ranged between 40 and 27.6 but the three positive samples showed clearer exponentially ascending fluorescence curves in the qPCR with ct-values of 23.13, 27.22 and 32.71, respectively. *Borrelia* DNA was detected in 1/19 (5%) *H. simplex* and in 2/174 (1%) *R. microplus* ticks (Table 3.1). Sequence analysis of a 1084 bp portion of 16S DNA showed 99.9% similarity of the first *R. microplus* sample (MN304841; Figure 3.3) with an uncultured *Borrelia* sp. from Ivory Coast, also detected in *R. microplus* (KT364342) and 99.7% identity to *Borrelia theileri* (KF569936). A *Borrelia* sp. from Madagascar detected in the blood of the lemur *Indri indri* equaled to 99.2% (KT722787). The *flaB* sequence (MN304843) analysis confirmed the close relationship to *B. theileri* (98.7%, 9/713 mismatches), and additionally revealed 100% identity to another Madagascan *Borrelia* sequence deposited in GenBank (KY070335; Figure 3.4). The closest match for the *gyrB* (MN304846) sequence was *Borrelia miyamotoi* with 91% (CP024351; Figure 3.5). There was no fitting *gyrB* sequence available for *B. theileri*. The second *Borrelia* sp. from *R. microplus* clustered together with *Borrelia turcica* (CP028884) and other tortoise associated but uncultured *Borrelia* spp. (see Figure 3.3 and Figure 3.5). In the *gyrB* sequence alignment this sample (MN304845) showed the highest similarity to a *Borrelia* sp. detected in tissue from a tortoise in Japan (AB473537; 95.8%), followed by 94.4% identity to *B. turcica* and 93.8% identity to a *Borrelia* sp. hosted by tortoise tick *Amblyomma geomydae* (AB529356; Figure 3.5). The corresponding values for the 16S sequence analysis (MN304842) were 99.6% (*B. turcica*) and 99.4% (*Borrelia* sp., *A. geomydae*), respectively (Figure 3.3). We were not able to get a *flaB* amplificate for this sample. Yet, the *flaB* amplification was the only successful for the *Haemaphysalis* DNA sample. Here, we obtained 97.3% identity of the 722 bp sequence (MN304844) to *Borrelia duttoni* (CP000976) and 97% identity to *Borrelia recurrentis* (CP000993; Figure 3.4).

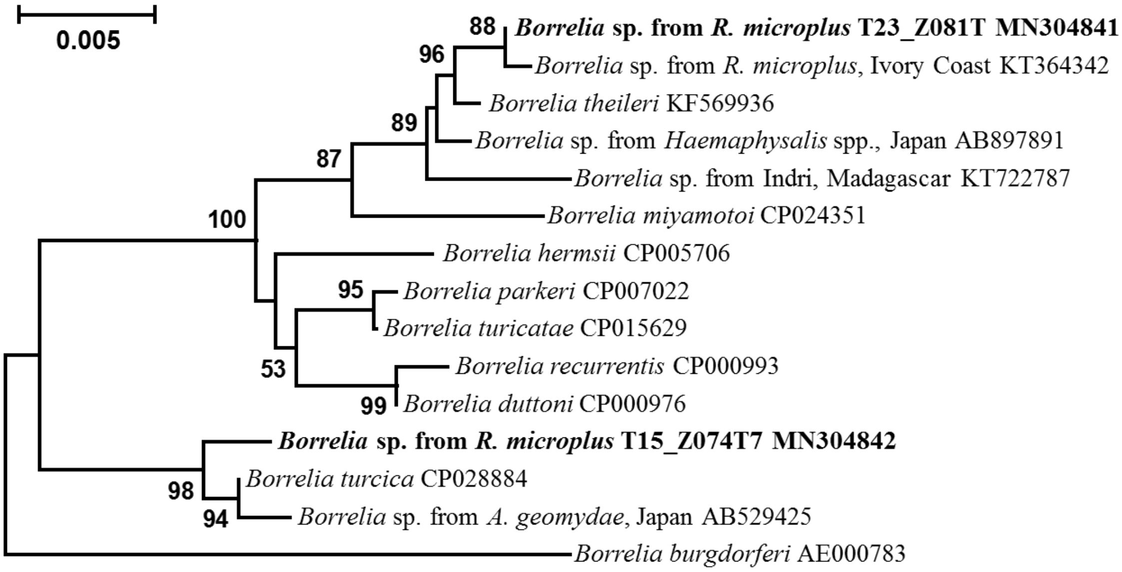

Figure 3.3. Evolutionary relationship of *Borrelia* spp. based on 16S rDNA alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.08247409 is shown. The bootstrap values >50 are shown next to the branches. The tree is drawn to scale. There were a total of 1083 positions in the final dataset.

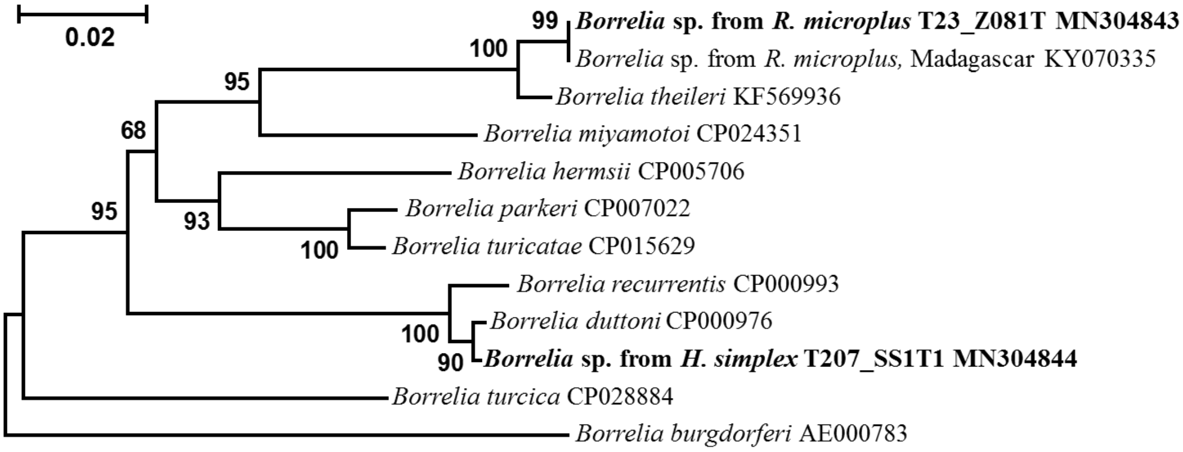

Figure 3.4. Evolutionary relationship of *Borrelia* spp. based on *flaB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.41705634 is shown. The bootstrap values are shown next to the branches. The tree is drawn to scale. There were a total of 704 positions in the final dataset.

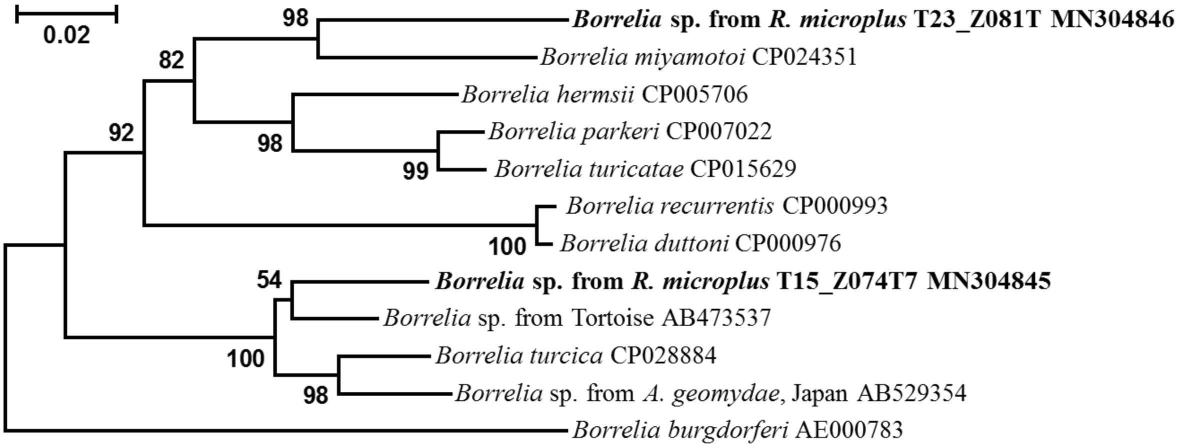

Figure 3.5. Evolutionary relationship of *Borrelia* spp. based on *gyrB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.61223084 is shown. The bootstrap values are shown next to the branches. The tree is drawn to scale. There were a total of 594 positions in the final dataset.

Bartonella screening

Neither *A. variegatum* nor any flea species DNA sample was screened positive by the *Bartonella* ITS qPCR. For louse pools we received 24 positive reactions, yet 11 of them had a ct-value above 40, three of which were confirmed by *gltA* or *rpoB* conventional PCR. 14/35 positive *H. quadripertusus* pools (MIR 8%) and 2/37 (MIR 2%) positive *L. verruculosus* pools were found finally (Table 3.3). Two different strains of *Bartonella* sp. were detected, one from *H. quadripertusus* and the other from *L. verruculosus*. Both *H. quadripertusus* associated PCR products (*rpoB*: MN304836; *gltA*: MN304840) showed 100% similarity to an uncultured *Bartonella* sp. from Israel (KJ522489; KJ522487), which was also recovered from *H. quadripertusus* (Figure 3.6). The species most related to this phylum was represented by *Bartonella chomelii* sharing 96.2% identical bases of *rpoB* sequence (KM215708) and 96.1% identity of *gltA* sequence (KM215692). The *rpoB* amplification of the *L. verruculosus* pools (MN304838) revealed 90.6% sequence identity with *Bartonella henselae* (BX897699) and 90.1% identity to a GenBank entry of *Bartonella quintana* (BX897700). The phylogenetic tree based on *gltA* sequences is not shown.

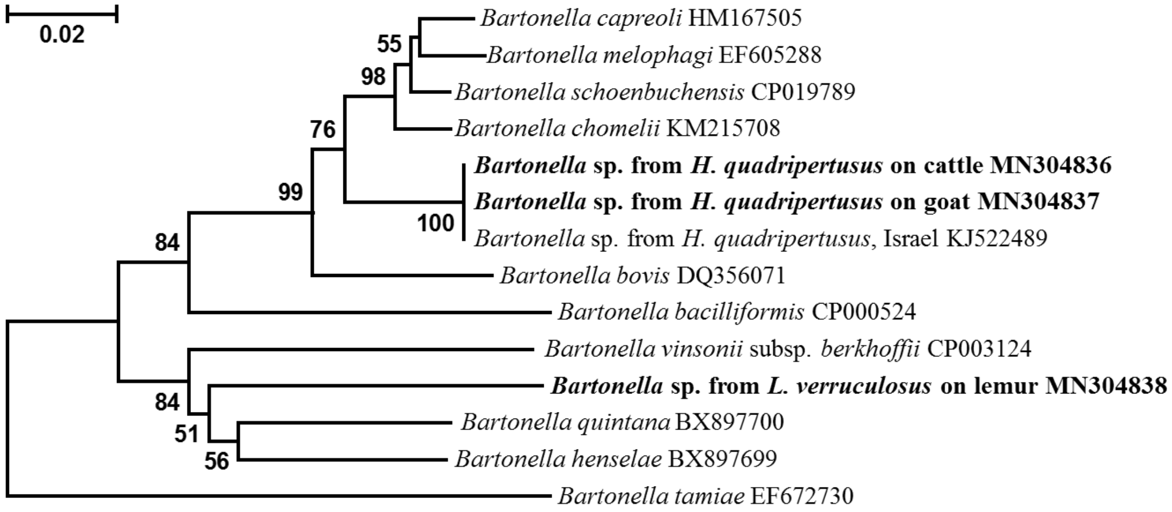

Figure 3.6. Evolutionary relationship of *Bartonella* spp. based on *rpoB* alignment. Sequences generated in the course of this study are displayed in bold. The optimal NJ-tree with the sum of branch length = 0.55666004 is shown. The bootstrap values >50 are shown next to the branches. The tree is drawn to scale. There were a total of 839 positions in the final dataset.

Yersinia pestis screening

The presence of *Y. pestis* DNA was proved in 4/76 flea samples (MIR 2%), comprising three pools of *E. gallinacea* and one pool of *X. cheopis*, by specific qPCR (Table 3.2). All four pools contained fleas collected from the same black rat (*R. rattus*) individual. Spleen and kidney DNA samples from this rat were also screened positive by qPCR. *Y. pestis* DNA was further detected in spleen samples of two other hosts, one mouse and one rat. However, ct-values where quite high (fleas: 38.84 – 45; kidney: 40.82; spleens: 34.93/39.58 [rats] and 40.59 [mouse]).

Co-infection

Among all investigated ectoparasite individuals or pools, seven were co-infected by rickettsiae and bartonellae (six *H. quadripertusus* and one *L. verruculosus*).

Discussion

Current changes in land use and the transformation of habitats bear the threat of yet unknown transmissions of pathogens between native and introduced animal species as well as between animals and humans. These transmissions can have fatal effects on all hosts affected (e.g., Ostfeld et al., 2005; Köndgen et al., 2017). The data presented here add analyses of possible bacterial pathogens to previous studies on the occurrence of arthropod ectoparasites on various animal species of southwestern Madagascar (Ehlers et al., 2016, 2019). While rickettsial DNA was detected in all species of arthropod hosts, findings of borreliae, bartonellae and *Y. pestis* were limited to only two ectoparasite species each. In the following paragraphs we review and discuss the addressed pathogens separated by genus.

Rickettsiae

The screening results presented in this study revealed a remarkably broad spectrum of ectoparasite species containing rickettsial DNA. Rickettsiae are Gram-negative obligate intracellular pathogens which reveal a remarkably wide range of arthropod hosts including bloodsucking ectoparasites (Weinert et al., 2009). Recent phylogenetic analyses subdivide this genus into four main groups, the ancestral, transitional, typhus (TG), and spotted fever group (SFG; Gillespie et al., 2008). SFG rickettsiae are predominantly transmitted by ticks and cause mild to severe forms of spotted fever (Fournier et al., 2009). The TG rickettsiae, *Rickettsia typhi* and *Rickettsia prowazekii*, are the agents of endemic (murine) and epidemic typhus, respectively. Whereas *R. prowazekii* is transmitted by the body louse (*Pediculus humanus corporis*; Parola et al., 2006), *R. typhi* is transmitted by fleas, usually *X. cheopis*, but also by other flea species and arthropod vectors (Azad, 1990). *Rickettsia felis*, a further flea-borne rickettsial pathogen, belongs to the transitional group and is transmitted mainly by the cat flea *C. felis*. As for *R. felis* the pathogenic potential of the other so called *R. felis*-like organisms (RFLOs) is still unclear and new species are described constantly (Jiang et al., 2013; Lopez-Velez et al., 2015; Mediannikov et al., 2015).

Each of the 13 species of ticks, fleas or lice was tested positive for rickettsial DNA (Tables 3.1-3.3). Compared to this broad diversity of host species, the diversity of *Rickettsia* spp. obtained from the phylogenetic analyses appeared relatively narrow. The majority of the DNA sequences could be assigned to *R. africae* (Figure 3.2), resembling 100% of the *ompB* sequence or holding one mismatch, respectively. Except *P. irritans* (n=10), all ectoparasite species provided *R. africae* DNA. *Rickettsia africae* is widely distributed on the African continent and has been detected in at least 13 hard tick species (Parola et al., 2013; Halajian et al., 2018) and one bird flea species (Sekeyová et al., 2012), but, to our knowledge, there are no further reports of detection of *R. africae* in soft ticks, louse or flea species so far. High prevalence of *R. africae* seems to be common for *A. variegatum* ticks. Previous studies in African countries found prevalences of *R. africae* beyond 50% in Nigeria and Uganda (Lorusso et al., 2013), the Union of the Comoros (Yssouf et al., 2014), Cameroon (Vanegas et al., 2018) and Côte d'Ivoire (Ehounoud et al., 2016). However, the situation in Madagascar seems to be special since this is the third study reporting 100% of a cohort of *Amblyomma* ticks being infected by *R. africae* (Keller et al., 2015; Ehlers et al., 2016). Due to transovarial and transstadial (vertical) transmission of *R. africae* in *A. variegatum* (Socolovschi et al., 2009), this phenomenon is consequential. Nevertheless, human infections by SFG rickettsiae are only anecdotally documented in Madagascar (Fournier et al., 2009). Lower prevalence, as discovered in Mozambique (5.6%; Matsimbe et al., 2017) or Nigeria (Ogo et al., 2012), for instance, seems to be an exception. Throughout the literature involving rickettsial burdens of *Rhipicephalus* spp. from cattle, prevalences are significantly lower, ranging from 0 to 10% (Ogo et al., 2012; Yssouf et al., 2014; Ehounoud et al., 2016; Vanegas et al., 2018). Again, our results from Madagascar depict another situation heading with 16% (28/176) of *R. microplus* containing *R. africae* DNA.

The other rickettsiae found in our study derived from fleas and were 100% homologous to *R. felis* (one pool of *C. felis*.) or clustered together with Candidatus *R. senegalensis* (Figure 3.2), which was first discovered in cat fleas (Mediannikov et al., 2015). Due to our findings we can now add three flea species (*E. gallinacea*, *X. cheopis* and *P. irritans*) to the list of potential hosts for this RFLO. *Rickettsia felis* has been associated with 12 flea species in continental Africa (Brown and Macaluso, 2016). Its principal vector, *C. felis*, was demonstrated to be capable of vertical transmission of *R. felis* (Wedincamp and Foil, 2002) and high prevalence of this bacterium (up to 95%) in central Africa seems to be common (Mediannikov et al., 2012; Leulmi et al., 2014). In Madagascar, *R. felis* had been detected in *X. cheopis* (2%) and in *P. irritans* fleas (26%; Rakotonanahary et al., 2017). The same study reports *R. typhi* infection of *X. cheopis*; and there are also reports of murine typhus in travelers acquired in Madagascar (Walter et al., 2012). Yet, we did not find *R. typhi* in our study.

Borreliae

Compared to *Rickettsia* spp., borreliae seem to be less prevalent in Madagascar. The genus *Borrelia* phylogenetically splits up into three main groups: Lyme disease (LD) borreliae, relapsing fever (RF) borreliae, and reptile-associated (REP) borreliae (Takano et al., 2010; Franke et al., 2012). LD is the most relevant tick-borne disease from the northern hemisphere, while in Sub-Saharan Africa pathogenic borreliae are represented by the RF group of the genus. An infection with *B. recurrentis*, transmitted to humans by *Pediculus humanus corporis*, is called epidemic or louse-borne relapsing fever (LBRF). In South and East Africa and Madagascar tick-borne relapsing fever (TBRF) is caused by *B. duttoni* transmitted by soft ticks of the *Ornithodorus moubata* complex. Yet, there are many more *Borrelia* spp., each transmitted by its specific soft tick vector worldwide, while some RF species such as *Borrelia lonestari*, *B. theileri* and *B. miyamotoi* are transmitted by hard ticks (Lee et al., 2014a; Talagrand-Reboul et al., 2018). Endemic RF has not been diagnosed in Madagascar since the early 1950s, probably due to the decline of the *O. moubata* s.l. vector population (Colas-Belcour et al., 1952; Rodhain and Fontenille, 1989). Epidemic RF has never been diagnosed in Madagascar, but seems to be emerging regularly in continental Africa (Elbir et al., 2013). Borrelioses of domestic and wildlife animals are comparably neglected in Madagascar despite the presence of their potential vectors. For example, the cattle tick *R. microplus* known to host *B. theileri* (Smith et al. 1978), a species causing bovine borreliosis, is well established over the whole country (Stachurski et al., 2013). *Borrelia* spp. was also detected in the blood of three lemur species (*Lepilemur mustelinus*, *Propithecus diadema* and *Indri indri*; Larsen et al., 2016; Qurollo et al., 2018).

In the course of our *Borrelia* screening, only three tick individuals were found to carry borreliae (Table 3.1). These were two out of 176 *R. microplus* (1%). Interestingly, one of them clustered together with the tortoise associated portion of the genus as confirmed by the 16S rDNA and *gyrB* alignment (Figure 3.3 and Figure 3.5). The other *Borrelia* DNA sample obtained from *R. microplus* was similar to *R. theileri* 16S rDNA (99.7%) and also showed 99.2% identity to borreliae detected in lemur blood (Larsen et al., 2016), indicating a high distribution of this pathogen. Hagen et al. (2018) detected the same borreliae in close proximity to our study site. The prevalence reported here is congruent to our results and these of other studies, which found *B. theileri* infecting less than 1% of *R. microplus* and 0.5% of *Rhipicephalus geigyi* ticks, respectively (Yparraguirre et al., 2007; McCoy et al., 2014). While *Haemaphysalis* spp. hosting borreliae are well known from east Asia (Lee et al. 2014b; Furuno et al., 2017; Khoo et al., 2017), our finding of *Borrelia* DNA in a *Haemaphysalis* tick is apparently a rarity regarding Africa. This sample showed 97.3% identity to *B. duttoni* (Figure 3.4), the causative agent of endemic RF in human. This could be a hint of the *Haemaphysalis* tick and perhaps its tenrec host maintaining the pathogen in absence of its main vector *O. moubata*.

Bartonellae

There is a very limited number of studies about the presence of *Bartonella* sp. in Madagascar, reporting the detection of *Bartonella* DNA in fruit bats and their ectoparasites (bat flies and fleas; Brook et al., 2015), in rats, and *Synopsyllus fonquerniei* fleas (Brook et al., 2017), in one *Haemaphysalis lemuris* tick (Lado et al., 2018) and in both head (Eremeeva et al., 2019) and body lice (Sangaré et al., 2014). Bartonellae are Gram-negative facultative intracellular bacteria whose vectors are known distinctively for only a few species: *Bartonella bacilliformis* (the agent of Oroya fever) is transmitted by sandflies of the genus *Lutzomyia* (Battistini, 1931), *B. henselae* (causative agent of cat scratch disease) is transmitted by *C. felis*, and *B. quintana* (the trench fever pathogen) by *P. humanus* (Billeter et al., 2008).

We found *Bartonella* infection of two louse species: The cosmopolitan cattle louse, *H. quadripertusus*, and *L. verruculosus* (Table 3.3), endemic in Madagascar and specialized in mouse lemurs (Ehlers et al., 2019). A potentially new *Bartonella* variant, closely related to other bartonellae infecting ruminants, was detected in 14/35 *H. quadripertusus* pools. As indicated by 100% identity of the amplified *rpoB* sequence (Figure 3.6), the same strain was detected earlier in Israel, also in *H. quadripertusus* (Gutiérrez et al., 2014). The *rpoB* sequence derived from *Bartonella* DNA isolated from 2/70 *L. verruculosus* pools interestingly revealed closest relationship to the two pathogenic *Bartonella* spp., *B. henselae* and *B. quintana*. Yet, there is only similarity of about 90% to both species, indicating that this *Bartonella* sp. might be a novel strain as well.

Ticks are under growing suspicion of being capable to transmit *Bartonella* spp., at least the genera *Dermacentor* and *Ixodes* (Angelakis et al., 2010a, 2010b). In Taiwan, *R. microplus* exhibited a *Bartonella* prevalence of 15.7% (Tsai et al., 2011). However, all ticks subjected to our *Bartonella* screening were tested negative (Table 3.1).

The same applies to fleas which seem to be commonly infected by bartonellae elsewhere (Sackal et al., 2008; Tsai et al., 2010; Fernandez-Gonzalez et al., 2016), but not at our study site (Table 3.2). Negative results are also presented from the Democratic Republic of the Congo, where 123 fleas were screened for bartonellae (Leulmi et al., 2014). These findings are coherent to the situation in Sub-Saharan Africa, where the occurrence of bartonellae is rare, or related studies have not been conducted (see reviews by Billeter et al., 2008; Tsai et al., 2011).

Yersinia pestis

Plague, caused by the Gram-negative bacterium *Y. pestis*, is undeniably the most notorious disease in Madagascar. The WHO (Bertherat, 2016) reported 3,248 human cases from 2010 to 2015 worldwide, including 584 deaths. Madagascar is by far the most affected country with 2,604 reported cases (476 deaths) attributed to pneumonic and bubonic plague and sepsis. From August to November 2017 Madagascar was faced with a plague epidemic of unusual size. The WHO (2017b) reported a total of 2,417 confirmed, probable and suspected cases of plague, with the majority (1,854) classified as pneumonic plague during this outbreak.

In Madagascar the persistence of plague is strongly connected to the highly invasive character of the black rat, which not only spreads in cities but also adapts well to rural areas and natural habitats (Duplantier and Rakotondravony, 1999). Not only *X. cheopis* is involved in the epidemic cycles in Madagaskar, but also the endemic flea *S. fonquerniei* (Duplantier and Duchemin, 2003). The contribution of *S. fonquerniei* is required to maintain sylvatic transmission cycles, because *X. cheopis*, the principal vector for humans, predominantly occurs in human housings (Duplantier and Duchemin, 2003). In 1998, plague re-emerged in the district of Ikongo between 250 m and 540 m of altitude (Duplantier et al., 2005), considerably below the postulated limit of Madagascar’s plague focus of 800 m (not including Mahajanga; Brygoo, 1966). After this outbreak four individuals of endemic small mammals were found to be seropositive for IgG anti-*Y. pestis* (Duplantier et al., 2001). The effect of involvement of a new reservoir host was demonstrated by the introduced *Suncus murinus* facilitating several outbreaks in the coastal city of Mahajanga in the 1990s (Duplantier and Duchemin, 2003).

Here we present the molecular detection of *Y. pestis* DNA in two flea species, *E. gallinacea* and *X. cheopis* (Table 3.2), and in the rat they were collected from, in a coastal region of Madagascar. As described above, the plague foci normally lie above 800 m, but under certain circumstances, this boundary could fall. However, the mentioned rat was trapped in a village, and samples from a forest site were all negative. Additionally, no endemic fleas have been found in the region.

Echidnophaga gallinacea was proven to become infected with *Y. pestis* in a laboratory study (Weyer, 1942). Yet, for its life history this flea species seems unsuitable for serving as a vector, because once it has bitten the host it stays attached, and it was observed that it would not leave even when the host died (Suter, 1964). In contrast to *E. gallinacea* the vector potential of *X. cheopis* is verified, but indicated by the high ct-value of the qPCR (>38) found in our study, the bacterial burden inside the fleas may have been insufficient for transmission.

Conclusion

In conclusion, we identified a broad range of vector-borne bacteria present in southwest Madagascar. In that particular rural region of southwest Madagascar, where humans live in close contact to livestock and rats, we found pathogenic bacteria circulating in ectoparasites which are also known to feed on humans (ticks and fleas). We illustrated a large distribution of rickettsiae among the ectoparasite fauna of the region. Borreliae and bartonellae were both found to be restricted to only two ectoparasite species each, which likely do not have the potential for interspecific transmission due to host specificity. The finding of *Y. pestis* DNA in fleas of a rat in a geographical region far off the known plague foci of Madagascar was unexpected.

Our results do not prove that the detected bacteria actually live and reproduce in or are transmitted by the examined arthropods. This limitation is due to the fact that our study was based on the mere detection of bacterial DNA. We also cannot explain the high distribution of *R. africae* that stands in contrast to the distinct host specificity of most infected arthropods. However, this indicates that these pathogens have crossed species boundaries in the past.

Future studies should address the epidemiological relevance of the vector-borne pathogens detected in our study. Seroprevalence studies for past infections as well as pathogen detection in acutely infected hosts, should be performed at the study site in order to demonstrate the resulting disease burdens in the local human population, livestock and the endemic fauna.

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Ethical approval: All applicable institutional and/or national guidelines for the care and use of animals were followed. The field work was approved by the ethics committee of the Institute of Zoology of Hamburg University before the initiation of this study and authorized by the Ministère de L'Environnement, de l'Ecologie, de la Mer et des Forêts. (Research permits: N°136/16/- and N°002/ 17/MEEF/SG/ DGF/DSAP/SCB.Re; export permit: N°345-17/MEEF/SG/DGF/ DREEFAAND/SFR). This article does not contain any studies with human participants performed by any of the authors.

Declaration of Competing Interest: none declared

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Supplementary material

Table S1. PCR conditions.

Bacteria genus	Gene/target	protein	Primer/ probe	Sequence 5' - 3'	ORF position	Amplicon size
Rickettsia	*ompB*	Outer membrane protein B	120-M59	CCGCAGGGTTGGTAACTGC	38-56	856 bp
			120-807	CCTTTTAGATTACCGCCTAA	874-893	
Bartonella	ITS		ITS-Bart-F	GGGGCCGTAGCTCAGCTG		169 bp
			ITS-Bart-R	TGAATATATCTTCTCT		
			ITS-Bart-probe	FAM-CGATCCCGTCCGGCTCCACCA-Tamra		
	rpoB	DNA-directed RNA polymerase subunit b	rpoB-1400F	CGCATTGGCTTACTTCGTATG	1435-1455	893 bp
			rpoB-2300R	GTAGACTGATTAGAACGCTG	2308-2327	
	gltA	Citrate synthase	Bhcs.781p	GGGGACCAGCTCATGGTGG	782-800	378 bp
			Bhcs.1137n	AATGCAAAAAGAACAGTAAACA	1139-1160	
Borrelia	16 S rDNA		ParBor16s3F	AGCCTTTAAAGCTTCGCTTGTAG	202-224	148 bp
			Par Bor16s3R	GCCTCCCGTAGGAGTCTGG	331-349	
			Par Bor16sP	6FAM-CCGGCCTGAGAGGGTGAACGG-BBQ	287-307	
			16RnaL	CTGGCAGTGCGTCTTAAGCA	38-57	1338 bp
			16RnaR	GTATTCACCGTATCATTCTGATATAC	1341-1366	
	gyrB	DNA gyrase subunit B	gyrB-343	GTTGGAATTTCAGTTGTTAATGC	343-365	1157 bp
			gyrB-1480	CCATCAACATCAGCATCAGC	1499-1480	
			gyrB 5'+1	TTATCAAAGAGACTTAGGGAACTTGC	547–572	1022 bp
			gyrB 3'+1	TGCCCATTCTCAATTAACTCCC	1547-1568	
			gyrB-F	TTAAAGGGCCTTGAAGCTGTTAG	31-53	641 bp
			gyrB-R	ACAAAAGCTTTTATTCCACCTTCA	648-671	
	flaB	Flagellin B	flaB-BOR1	TAATACGTCAGCCATAAATGC	15-35	777 bp
			flaB-BOR2	GCTCTTTGATCAGTTATCATTC	770-791	
Yersinia	PCP1 plasmid/ 3' UTR of *pla*	Coagulase/fibrinolysin (Plasminogen activator)	YpplaS1	GCGCTCATTCTGTTGTTTTGC		77 bp
			YpplaAs1	CGTTCCATGTCTAATTTGACCTTCT		
			YpplaP	FAM-TTGACATTCTCCACGTCTTTCAGGGCA-BHQ1		

Table S2. BLAST results

seq#	accession	organism	gene	length	vector	vector group	host
1	MN304836	*Bartonella*	*rpoB*	839	*Haematopinus quadripertusus*	louse	cattle
2	MN304837	*Bartonella*	*rpoB*	839	*Haematopinus quadripertusus*	louse	goat
3	MN304838	*Bartonella*	*rpoB*	839	*Lemurpediculus verruculosus*	louse	*Microcebus griseorufus*
4	MN304839	*Bartonella*	*gltA*	337	*Lemurpediculus verruculosus*	louse	*Microcebus griseorufus*
5	MN304840	*Bartonella*	*gltA*	337	*Haematopinus quadripertusus*	louse	cattle
6	MN304841	*Borrelia*	*16S rDNA*	1084	*Rhipicephalus microplus*	tick	cattle
7	MN304842	*Borrelia*	*16S rDNA*	1084	*Rhipicephalus microplus*	tick	cattle
8	MN304843	*Borrelia*	*flaB*	713	*Rhipicephalus microplus*	tick	cattle
9	MN304844	*Borrelia*	*flaB*	722	*Haemaphysalis* sp.	tick	*Setifer setosus*
10	MN304845	*Borrelia*	*gyrB*	594	*Rhipicephalus microplus*	tick	cattle
11	MN304846	*Borrelia*	*gyrB*	594	*Rhipicephalus microplus*	tick	cattle
12	MN304847	*Rickettsia*	*ompB*	789	*Ctenocephalides felis*	flea	dog
13	MN304848	*Rickettsia*	*ompB*	783	*Echidnophaga gallinacea*	flea	dog
14	MN304849	*Rickettsia*	*ompB*	783	*Ctenocephalides felis*	flea	cat
15	MN304850	*Rickettsia*	*ompB*	783	*Echidnophaga gallinacea*	flea	cat
16	MN304851	*Rickettsia*	*ompB*	783	*Echidnophaga gallinacea*	flea	*Galidictis grandidieri*
17	MN304852	*Rickettsia*	*ompB*	783	*Echidnophaga gallinacea*	flea	chicken
18	MN304853	*Rickettsia*	*ompB*	783	*Echidnophaga gallinacea*	flea	*Mus musculus*
19	MN304854	*Rickettsia*	*ompB*	789	*Xenopsylla cheopis*	flea	*Mus musculus*
20	MN304855	*Rickettsia*	*ompB*	789	*Xenopsylla cheopis*	flea	*Rattus rattus*
21	MN304856	*Rickettsia*	*ompB*	783	*Echidnophaga gallinacea*	flea	*Rattus rattus*
22	MN304857	*Rickettsia*	*ompB*	789	*Pulex irritans*	flea	off-host
23	MN304858	*Rickettsia*	*ompB*	783	*Amblyomma variegatum*	tick	cattle
24	MN304859	*Rickettsia*	*ompB*	783	*Rhipicephalus microplus*	tick	cattle
25	MN304860	*Rickettsia*	*ompB*	783	*Amblyomma chabaudi*	tick	*Macrotarsomys bastardi*
26	MN304861	*Rickettsia*	*ompB*	783	*Haemaphysalis sp.*	tick	*Setifer setosus*
27	MN304862	*Rickettsia*	*ompB*	783	*Argas echinops*	tick	*Echinops telfairi*
28	MN304863	*Rickettsia*	*ompB*	783	*Linognathus africanus*	louse	goat
29	MN304864	*Rickettsia*	*ompB*	783	*Lemurpediculus verruculosus*	louse	*Microcebus griseorufus*
30	MN304865	*Rickettsia*	*ompB*	783	*Linognathus vituli*	louse	cattle
31	MN304866	*Rickettsia*	*ompB*	783	*Haematopinus quadripertusus*	louse	cattle

Table S1. (continued)

PCR type	Reference	Detection limit	Positive control	T_A	Enzyme	Reaction volume	Taq µL	MgCl2	dNTP	Primer	Probe	DNA
conventional	Roux & Raoult 2000		*R. africae* gen. DNA	58°C	Platinum Taq	25 µL	0.15 µL	1.5 mM	0.2 mM	0.4 µM		2 µL
real time	own Lab	1-7 copies/ reaction	linearized vector with PCR-product	60°C	Quantitect	20 µL				0.4 µM	0.2 µM	2 µL
conventional	Renesto et al. 2001		*B. henselae* gen. DNA	53°C	Platinum Taq	25 µL	0.15 µL	1.5 mM	0.2 mM	0.4 µM		2 µL
conventional	Norman et al. 1995		*B. henselae* gen. DNA	54°C	HotStarTaq	25 µL	0.15 µL	2 mM	0.2 mM	0.6 µM		2 µL
real time	Parola et al. 2011		linearized vector with PCR-product	60°C	Platinum Taq	20 µL*	0.16 µL	4 mM	0.2 mM	0.6 µM	0.2 µM	4 µL
conventional	Barbour et al. 1996		Mo063b gen. DNA (Hagen et al. 2018)	54°C	Platinum Taq	25 µL	0.15 µL	1.5 mM	0.2 mM	0.4 µM		2 µL
conventional	own Lab	not tested	Mo063b gen. DNA (Hagen et al. 2018)	56°C	Platinum Taq	25 µL	0.15 µL	1.5 mM	0.2 mM	0.4 µM		2 µL
conventional	Schwan et al. 2005		Mo063b gen. DNA (Hagen et al. 2018)	56°C	Platinum Taq	25 µL	0.15 µL	1.5 mM	0.2 mM	0.4 µM		2 µL
conventional	own Lab	not tested	Mo063b gen. DNA (Hagen et al. 2018)	60°C	HotStarTaq	25 µL	0.15 µL	2 mM	0.2 mM	0.6 µM		2 µL
conventional	Assous et al. 2006		Mo063b gen. DNA (Hagen et al. 2018)	56°C	Platinum Taq	25 µL	0.15 µL	1.5 mM	0.2 mM	0.4 µM		2 µL
real time	Panning unpubl.	12.35 copies/ reaction	linearized vector with PCR-product	60°C	HotStarTaq Plus	20 µL	0.12 µL	3 mM	0.2 mM	0.4 µM	0.2 µM	2 µL

*including 0.04 µM BSA

Table S2. (continued)

1st hit	source	identity %	mis-matches	gaps	2nd hit	source	identity %	mis-matches	gaps
KJ522489	*H. quadripertusus*, Israel	100	0	0	KM215708	*B. chomelii*	96.2	31	1
KJ522489	*H. quadripertusus*, Israel	100	0	0	KM215708	*B. chomelii*	96.2	31	1
BX897699	*B. henselae*	90.6	79	0	BX897700	*B. quintana*	90.1	83	0
EF616717	*Polygenis gwyni*	89.0	37	0	CP003124		87.7	41	0
KJ522487	*H. quadripertusus*, Israel	100	0	0	KM215692		96.1	13	0
KT364342	Ivory Coast	99.9	1	0	KF569936	*B. theileri*	99.7	3	0
CP028884	*B. turcica*	99.6	4	0	AB529425	*A. geomydae*	99.5	6	0
KY070335	*R. microplus*, Madagascar	100	0	0	KF569936	*B. theileri*	98.7	9	0
CP000976	*B. duttonii*	97.3	8	2	CP000993	*B. recurrensis*	97.0	10	2
AB473537	Tortoise	95.8	25	0	CP028884	*B. turcica*	94.4	33	0
CP024351	*B. miyamotoi*	91.0	53	0					
CP000053	*R. felis*	100	0	0					
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	100	0	0	CP003341	*R. parkeri*	98.6	11	0
KU499848	*R. senegalensis*	98.4	4	1	CP000053	*R. felis*	96.2	30	0
KU499848	*R. senegalensis*	98.4	4	1	CP000053	*R. felis*	96.2	30	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
KU499848	*R. senegalensis*	98.4	4	1	CP000053	*R. felis*	96.2	30	0
CP001612	*R. africae*	100	0	0	CP003341	*R. parkeri*	98.6	11	0
CP001612	*R. africae*	100	0	0	CP003341	*R. parkeri*	98.6	11	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	100	0	0	CP003341	*R. parkeri*	98.6	11	0
CP001612	*R. africae*	100	0	0	CP003341	*R. parkeri*	98.6	11	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	99.7	2	0	CP003341	*R. parkeri*	98.3	13	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0
CP001612	*R. africae*	99.9	1	0	CP003341	*R. parkeri*	98.5	12	0

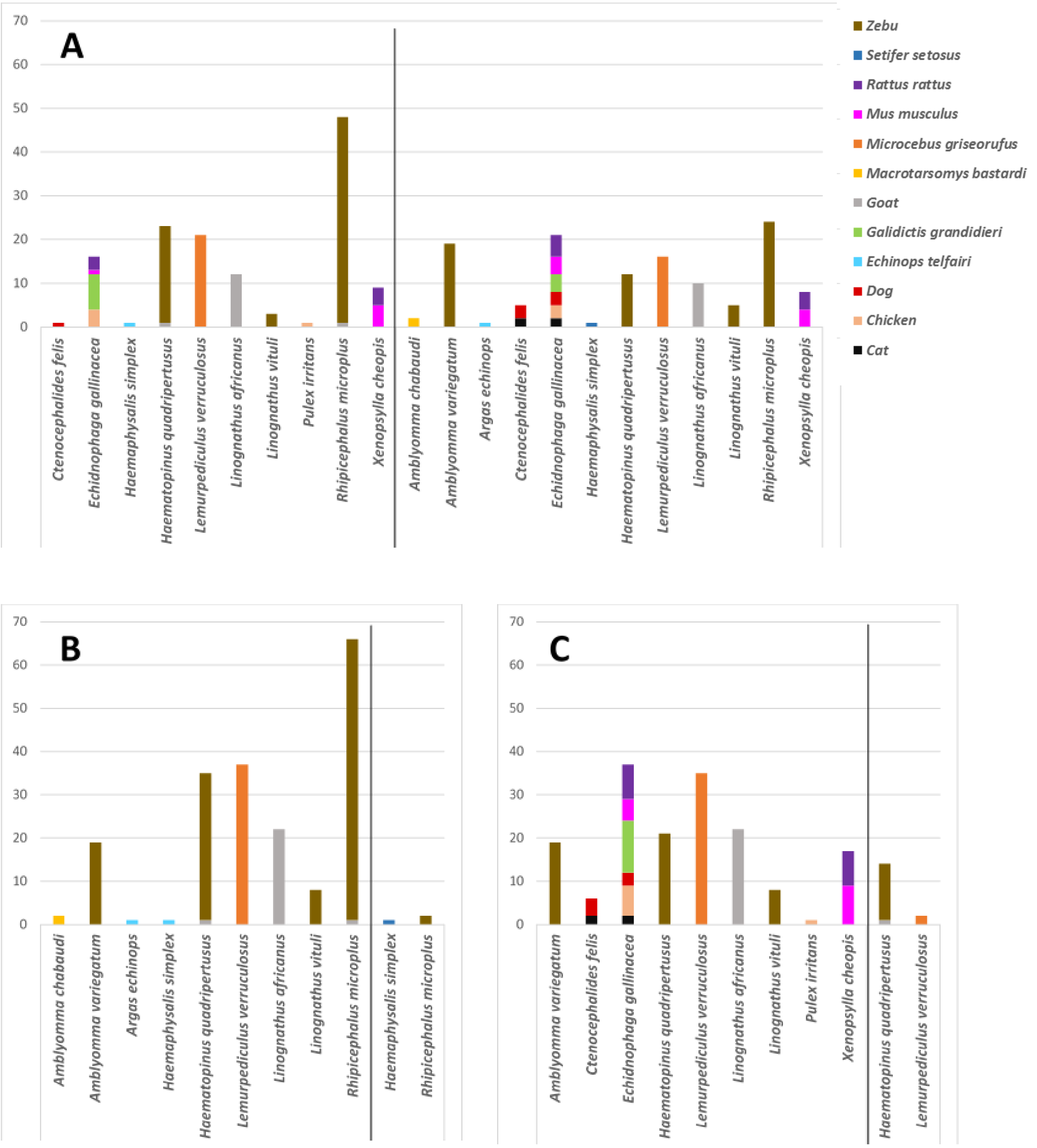


Figure S1. Bacterial screening. The bars indicate the number of host individuals harboring infected (right of the vertical line) or not infected (left of the vertical line) ectoparasites. A *Rickettsia*; B *Borrelia*; C *Bartonella*.

Author contribution

I hereby confirm that Julian Ehlers designed the study and conducted sampling in the field. He performed the experiments in the laboratory, evaluated the data and wrote the manuscript.

Jörg Ganzhorn

Prof. Dr. Jörg Ganzhorn

Chapter 4

General discussion

The risk of emerging diseases increases with human encroachment into previously largely untouched areas, by creating contact zones for people and their associated animals with the endemic wildlife (Daszak et al., 2000; Gortazar et al., 2014). As vectors of many pathogens ectoparasites represent an important route for disease transmission. This thesis investigates the abundance of possible vectors and vector-borne bacteria in an emerging disease hotspot (Brito et al., 2012; Jones et al., 2008).

In **Chapter 2** I revealed a first comprehensive overview of ectoparasites found on human associated animals on the one hand and in the wild small mammal community including a lemur and a mongoose species on the other hand. Overall, 17 different species of ticks, lice, fleas and mites have been registered during screening of 13 endemic and domestic host species.

In **Chapter 3** I demonstrated the presence of bacteria with zoonotic potential, *Rickettsia* spp., *Borrelia* spp., *Bartonella* spp. and *Yersinia pestis* which tend to infect a broad variety of mammals, potentially vectored by the collected Ectoparasites. While rickettsial DNA was detected in all species of arthropod hosts, findings of borreliae, bartonellae and *Y. pestis* were limited to two ectoparasite species each.

Presence of ectoparasites infesting endemic mammals

The most constantly trapped *Microcebus griseorufus* was infested by the sucking louse *L. verruculosus* occurring on 37 of 102 host individuals trapped inside Tsimanampetsotsa National Park (TNP) and the adjacent coastal zone. Three species of sucking lice are known to parasitize *Microcebus* spp., belonging to the genus *Lemurpediculus. L verruculosus* first description based on lice from a not clearly identified "mouse lemur" (Ward, 1951). It is known from *Microcebus rufus* in Ranomafana (Durden et al., 2010). *L. verruculosus* was also recovered from *M. griseorufus* at the Beza Mahafaly Special Reserve (BMSR) located about 100 km northeast of our study site (Rodriguez et al., 2012). The other louse species are known from Ankarafantsika National Park (ANP): *Lemurpediculus madagascariensis* was recently described as parasite of *M. murinus*, and the third species is infesting *Microcebus ravelobensis*, but is yet undescribed (Durden et al., 2018; Kiene et al., 2020).

Ticks and mites were completely absent from *M. griseorufus* trapped at the study site, which contrasts the reports of other regions. At BMSR *M. griseorufus* is commonly infested by the tick species *Ixodes lemuris*, *Haemaphysalis lemuris* and possibly *H. simplex* (Blanco et al., 2013; Rodriguez et al., 2012). Accordingly, *H. lemuris* infests ring-tailed lemurs (*Lemur catta*) at BMSR (Loudon et al., 2006), but was not found at Tsimanampetsotsa (Dutton et al., 2003). In ANP, Northern Madagascar, a fourth tick species, the recently discovered *Haemaphysalis microcebi,* infests both the sympatric mouse lemurs *M. ravelobensis* and *M. murinus*. Furthermore, each of the two lemur species is infested by three mite and one louse species in ANP (Kiene et al., 2020; Klein et al., 2018).

The only endemic rodent trapped during this study, *Macrotarsomys bastardi*, was found twice infested by *Amblyomma. chabaudi,* so far considered as a strictly host specific tortoise tick (Ehlers et al., 2016). Reports of ectoparasites infesting this rodent are almost absent as this species is rarely trapped (Youssouf and Rasoazanabary, 2008). Only two *Haemaphysalis* ticks have been associated with *M. bastardi* in the past (Uilenberg et al., 1979). But with only seven *M. bastardi* individuals trapped it is not suggestible if this infestation is common or accidental.

In the Tsimanampetsotsa region we collected *H. simplex* from the two hedgehog tenrec species *Setifer setosus* and *Echinops telfairi,* which are the preferred hosts according to Uilenberg et al. (1979), but neither on rodents nor on mouse lemurs. However, it had also been occasionally recovered from lemurs, *Propithecus verreauxi* (Uilenberg et al., 1979) and *M. griseorufus* (Rodriguez et al., 2012). This tick species is most likely nidiculous and can be found in small mammal nests. (Uilenberg et al., 1979).

We found *Galidictis grandidieri* being infested by introduced sticktight fleas (*Echidnophaga gallinacea*). This carnivore has never been subject of ectoparasite studies according to the Global Mammal Parasite Database (Nunn and Altizer, 2005). Since the distribution of *G. grandidieri* borders on the vicinity of human settlements (Marquard et al., 2011), it could be infected by fleas when ranging in search of food, or *E. gallinacea* could be brought to their habitat by straying dogs or feral cats. This is the only instance of an ectoparasite shared by both introduced and endemic mammals proved by this study. The demonstrated infestation of *G. grandidieri* with introduced fleas must be monitored carefully since this species is one of the most endangered carnivores in the world, and it is also the rarest of Madagascar's carnivores most likely becoming extinct (Cartagena-Matos et al., 2017, Hawkins, 2015).

Ectoparasites of Rattus rattus and detection of Yersinia pestis

Eight out of eleven *Rattus rattus* individuals (black rat, hereinafter also termed rat) trapped in houses were infested by *Xenopsylla cheopis*. *Echidnophaga gallinacea* was found infesting the same proportion of this rat sample. Both species were also hosted by mice (*Mus musculus*) captured in the same houses. The coexistence of mice could be an important factor in maintaining flea populations of villages. In contrast to the human commensal rats, the free-ranging rats trapped in agricultural habitats or in TNP were infested by mites (*Ornithonyssus bacoti*), and one single *Hoplopleura* louse, but did not host any fleas.

In ANP black rats are infested by *H. microcebi* ticks, *Polyplax* and *Hoplopleura* lice and two different species of mites (laelaptid and trombiculid taxa, respectively) (Kiene et al., 2020). O'Connor (2003) listed five species of mites infesting rats, and seven tick species have been recovered from rats across the country (Uilenberg et al., 1979). Moreover six flea species of the genus *Paractenopsyllus* are known to parasite black rats in rain forests of the central highlands or the northern mountains of Madagascar (Hastriter and Dick, 2009), and also *Synopsyllus fonquerniei* is found rather at higher altitudes, probably restricted southwards by increasing aridity (Duchemin, 2003).

One of these rats was tested positive for *Y. pestis* by PCR, as were both flea species *X. cheopis* and *E. gallinacea* collected from it. In laboratory experiments *E. gallinacea* was proven to receive *Y. pestis* and even transmit the pathogen when actively put on a new host (Burroughs, 1947). Yet, this flea supposedly has no vector function, as it feeds attached to the host similar to a tick, and tends to remain even on dead hosts (Pollitzer, 1954).

X. cheopis, on the other hand, is the most important vector of *Y. pestis* in Madagascar (Chanteau, 2006). There is not only *X. cheopis* involved in plague circulation in Madagascar, but also the endemic flea *S. fonquerniei* spreads the bacterium among rat populations with even greater vector efficiency (Andrianaivoarimanana et al., 2013; Duplantier and Duchemin, 2003). *Xenopsylla cheopis* multiplies inside houses, while *S. fonquerniei* is known from rodents dwelling outside human settlements. In Madagascar the persistence of plague is strongly connected to the highly invasive character of the black rat, which not only spreads in cities but also adapts well to rural areas and natural habitats, where it is augmenting the host spectrum of *S. fonquerniei* (Duplantier and Rakotondravony, 1999). The ability of *S. fonquerniei* to feed on both rats and endemic rodents explains the suspected transmission to endemic rodents and the persistence of sylvatic plague cycles (Duplantier and Duchemin, 2003; Duplantier et al., 2005).

The postulated limit of Madagascar’s plague foci lies above 800 m, strongly connected to the distribution of *S. fonquerniei* (Brygoo, 1966), but a plague outbreak in 1998 took place in the district of Ikongo (Duplantier et al., 2005), which lies at an altitude of between 250 and 540 m, suggesting that there could be other factors involved, possibly nonconventional flea vectors and endemic small mammals (Andrianaivoarimanana et al., 2013).

The forest-dwelling rats trapped in TNP, however did not host any fleas, nor did the endemic tenrecs or *M. bastardi*, a circumstance that inhibits the development of sylvatic plague foci. The detection of *Y. pestis* in *X. cheopis* fleas, on the other hand, together with the fact that these fleas were found in close proximity to humans is of high concern. However it can be doubted that the fleas can sustain their vector capacity under the regionally prevailing climatic conditions, since at temperatures above 27 °C the *Y. pestis* biofilm in the flea's foregut, a major principle of *Y. pestis* transmission, disappears (Gage, 2012).

Contribution of species introduction to the ectoparasite burden of endemic mammals

The introduction of alien species can have an enormous impact on native species and the environment (Vitousek, 1996; Vitousek et al., 1997). Contrary to the dilution effect, invasive species can add an "amplification effect" to disease dynamics of host communities by serving as pathogen reservoirs themselves and by competing and replacing native mammals. (Keesing et al., 2006). Rats are likely the most detrimental invaders (see Chapter 1), but also dogs and cats enter natural habitats and not only compete with or even pray on the endemic wildlife (Brockman et al., 2008; Sauther et al., 2011), but they could also transfer ectoparasites from human settlements to forests. For example, dogs in Madagascar have been recognized carrying multiple species of ticks: *Rhipicephalus sanguineus*, *R. microplus*, *A. variegatum*, *Otobius megnini*, all also introduced, and *Haemaphysalis obtusa* and (Uilenberg et al., 1979). The cats and dogs examined in villages of the study site, harbored no ticks, but were highly infested with fleas, *C. felis* and *E. gallinacea*, which makes them the suspected bridging species for *E. gallinacea* found among *G. grandidieri* inside TNP.

A further biological pollution has hit Madagascar in the course of livestock trade. Not only the ruminants themselves can stress the ecosystems, for example by overgrazing (Ratovonamana et al., 2013), also many ectoparasites, that have been introduced to Madagascar together with cattle, sheep and goats, (Barre and Uilenberg, 2010; Uilenberg, 1964) may cause damage to the endemic wildlife. Compared to the some 20 species of ixodid ticks infesting livestock under similar climatic conditions in southern and eastern Africa (Walker et al., 2003), it is remarkable that only *A. variegatum* and *R. microplus* seem to be established on cattle in Madagascar. In continental Africa, large ruminants including cattle are mostly infested by adult life stages of *A. variegatum*, while medium and large mammals as well as birds are frequent hosts of nymphs. Larvae are predominantly feeding on birds and small mammals (Hoogstraal, 1956).

The potential threat of tick-borne diseases to the Malagasy population, especially to pastoralists, is increased by underestimation or simply by the lacking awareness of the role of ticks in disease transmission. Bardsley and Thrusfield (2014) assessed by interviewing that cattle herders did not understand the association between disease and vector, and that they would not remove ticks they saw attached to their animals.

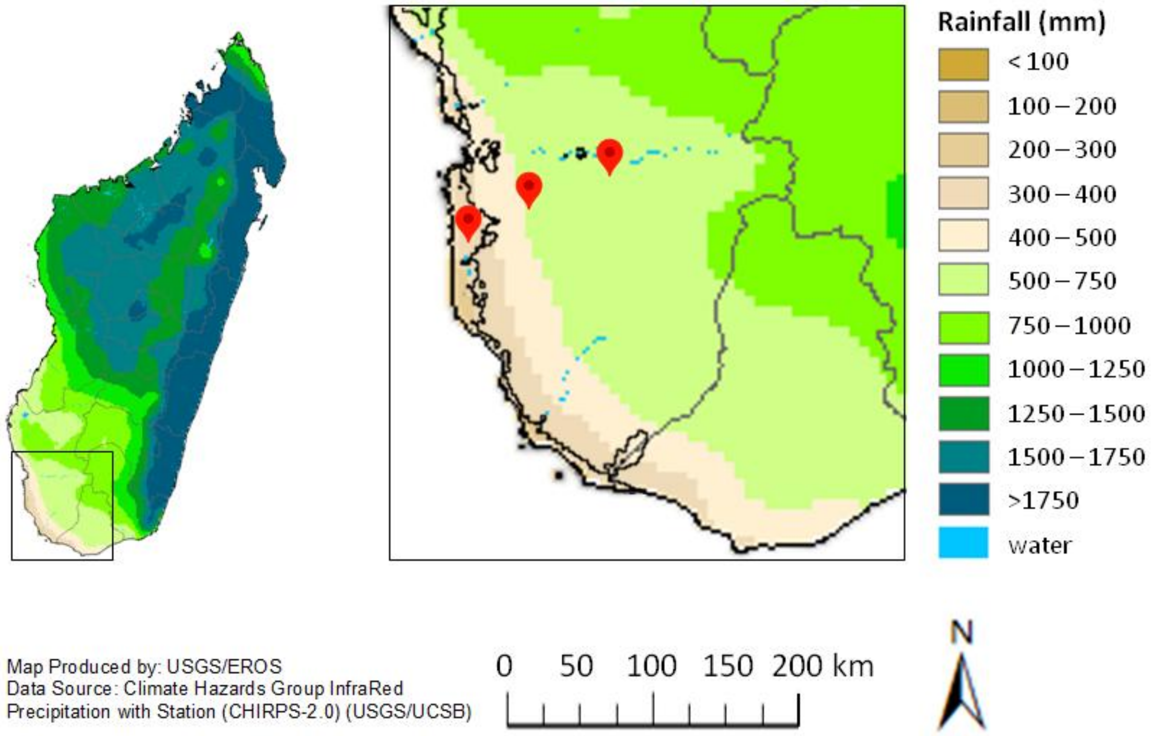
Seasonal variation in the occurrence of ectoparasites

The Malagasy livestock husbandry is dictated by the availability of food and water. An annual movement of livestock towards the Mahafaly plateau (transhumance) starts in November or December, when the food in the littoral is scanty and its quality is low, but the first rainfalls promise productive savannah vegetation on the higher plateau. The plateau receives more rain (400-450 mm/year instead of 300-350 mm/year (Goetter, 2016; Figure 4.1) and provides enough food, but the soils are water permeable and there are only a few ponds providing water. When the precipitation declines, around March to April, the herders are forced to drive their cattle back to the coast, where groundwater is available throughout the year (Feldt and Schlecht, 2016; Goetter, 2016; Ratovonamana et al., 2013).

In the frame of this study the transhumance hindered a systematic assessment of parasite burdens of livestock in the coastal zone during the rainy season. A small number of cattle that had not joined the transhumance were examined occasionally. This sample was not infested by any ticks, but it was noticed that cattle which had just returned from the plateau were infested. In this context it also seems noteworthy that during the rainy season study period, in the littoral ticks were frequently seen on oxen that are used as draft animals for transport of goods from the plateau (pers. obs.). Local stockbreeders from the study site claim that cattle are in better health condition in the littoral compared to the plateau (Ganzhorn, pers. comm.). The transhumance is also linked to decreasing animal health towards the end of the rainy season. After returning to the coastal plain the cattle are observed to recover. Although this observation may have several causes, the coincidence with the loss of ticks should be kept in mind.

Ectoparasite prevalence and intensity is supposed to be higher when environmental abiotic conditions are favorable (Altizer et al., 2006), most importantly for preventing desiccation, the greatest threat to survival. It is known that louse survival and development is limited by temperature (Matthysse, 1944), that moister climate conditions promote flea survival and reproduction (Parmenter et al., 1999), and humidity is also claimed to be the most limiting factor of tick survival (Gray et al., 2009; Needham and Teel, 1991). Regarding ticks, it must be noted that life cycles per se are adapted to seasonal conditions. Different life stages of ticks may occur on different hosts in certain periods of the year (Yawa et al., 2018).

A strong seasonal pattern has been observed in louse infestation of *M. griseorufus*. Half of the lemurs trapped in the dry season was infested by *L. verruculosus*, compared to only a quarter of individuals being infested in the wet season. In ANP louse prevalence of mouse lemurs was also higher in the dry season, peaking in October (Klein et al., 2018). This is consistent with observations in other tropical regions,where louse populations of livestock decline in the hottest months and peak in the cold, dry season (Gabaj et al., 1993; Kumar et al., 1994; Rashmi and Saxena, 2017).



Map Produced by: USGS/EROS
Data Source: Climate Hazards Group InfraRed Precipitation with Station (CHIRPS-2.0) (USGS/UCSB)

Figure 4.1: Average annual rainfall between 2001 and 2015. The red dots indicate the location of the study site in the coastal zone (left), the area on the Mahafaly plateau, where cattle are driven at the beginning of the rainy season (middle), and the location of BMSR (right). The black line along the coast is the 100 m - contour line (Wilmé et al., 2006) marking the border of the plateau.

Rainfall is supposed to be the key factor determining the life cycle of ticks so that one rainy season will lead to the completion of one life cycle (Petney et al., 1987). A study from Gambia reports that adult *A. variegatum* ticks infest cattle in the rainy season, and almost disappear in the dry season (Mattioli et al., 1997). In continental Africa, this species is supposed to be limited by arid conditions, i.e. below 400 mm annual rainfall (Barré and Uilenberg, 2010). The virtual absence of cattle ticks in the littoral zone of the Tsimanampetsotsa region is most likely due to aridity (Figure 4.1).

At BMSR immature stages of *H. lemuris* seem to occur only in the dry season, then infesting *M. griseorufus* (Rodriguez et al., 2015; Figure 4.2), whereas adult ticks infest sifakas and ring-tailed lemurs year-round (Loudon et al., 2006). Seasonal occurrence of juvenile ticks on *Microcebus spp*. is also observed in ANP, where larvae are found from May to July and nymphs from June to November. (Klein et al., 2018). Year-round infestation by adult *H. lemuris* of *Propithecus diadema* is reported from a rain forest habitat (Klompen et al., 2015). As both host species required for the different life stages, *M. griseorufus* and *L. catta*, occur in TNP, *H. lemuris* is supposedly lacking for another reason. This species possibly reaches its distribution limit at the changing from the Mahafaly Plateau to the coastal plain, similar to the cattle ticks, due to decreasing humidity. It is also possible that vegetation cover and the understory of the littoral forest is insufficient to avoid desiccation of eggs or larvae (Tukahirwa, 1976). This restriction would apply equally to all tick species that occur on the plateau but were not found in the littoral. In line with this assumption, Yawa et al. (2018) also did not find any *R. microplus* ticks in a South African region with an annual rainfall of 350 mm. Mites are documented with unvarying prevalence around the year (Klompen et al., 2015; Wright et al., 2009), nevertheless, the result that *M. griseorufus* did not show any sign of mite infestation, was unexpected.

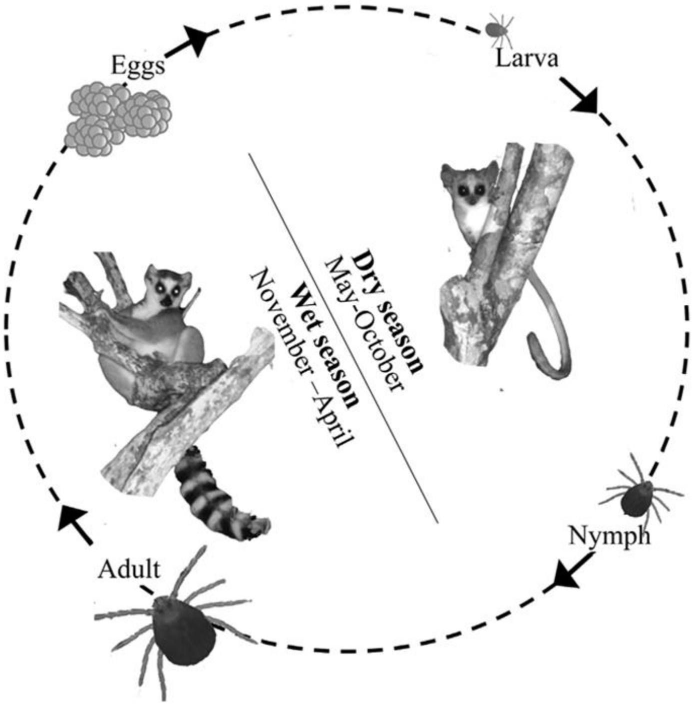


Figure 4.2: Life cycle of *H. lemuris* at BMSR, hypothesized by Rodriguez et al. (2015), where juvenile ticks are found on *M. griseorufus* and adults on *L. catta* depending on season (adapted from the cited reference).

There are some barriers for systematic assessment of seasonal effects, as the method of trapping itself can be biased by season. Many of Madagascar's mammal species are capable of physiological mechanisms to react to food deprivation primarily during the dry season. These species can undergo periods of inactivity, lasting from a few days to several months, with their body temperature passively adapted to the ambient temperature, in order to save energy spent by active thermoregulation (Nowack et al., 2020). So-called heterotherms are represented by *M. griseorufus* and the tenrec species of the study site (Kobbe et al., 2011; Levesque and Lovegrove, 2014; Lovegrove and Genin, 2008). When being in a state of torpor or hibernation the respective animals consequently will not be trapped. A sufficient natural supply of food in the rainy season can also diminish trapping success by reducing the relative attractiveness of the bait inside the trap. Pitfall traps might be more appropriate for trapping of tenrecs (Goodman et al., 1996), but we rejected installing pitfall traps in order to prevent trapped animals from being exposed to predators and scorpions. Furthermore, the utilized box traps may be too small for larger specimens, precluding *Tenrec ecaudatus* or adult *Setifer setosus*. Also the choice of the bait is not necessarily suitable for all target animals equally. The last two points cannot be evaluated afterwards. But as tenrecs were only trapped in the rainy season, their behavior of energy saving does obviously matter. However, there was no seasonal bias of trapping success observed in *M. griseorufus*. Overall, compared to other studies (Rasoazanabary, 2011; Youssouf and Rasoazanabary, 2008) trapping rate was high.

Habitat disturbance and other factors influencing ectoparasite abundance

Nevertheless, most species were not caught in sufficient numbers to allow statistical analysis between habitats of different quality, but *M. griseorufus* was trapped in reliable numbers. Lemurs captured inside TNP (high quality) revealed the highest louse prevalence (52%) followed by the most disturbed habitat (low quality, 42%), while the habitat of medium quality ranged in between (20%). This result contrasts with the assumption that habitat degradation leads to higher ectoparasite prevalence (Junge et al., 2011; Wright et al., 2009). Other factors likely have stronger influence on the prevalence, which are discussed below.

Depending on habitat quality, the density of a host population is crucial for survival of ectoparasite populations. A higher host density would mean a higher possibility for the parasite to get in contact with a suitable host. Habitat quality can positively influence population density by offering stable food resources (Balko and Brian Underwood, 2005). On the other hand, also poor quality habitats can mediate density, when populations are crowded by fragmentation their density would at least initially rise (Debinski and Holt, 2000). The density proxy of mouse lemurs we calculated for the different habitats (animal captures per 100 trap nights) rose with habitat quality (low: 2.5; medium: 3.5; high: 3.8), explaining the higher louse prevalence in the pristine forest following an increasing density, but giving no illustration for the decline of prevalence from low to medium quality. A recent study (Kiene et al., 2020) found edge effects playing a role in ectoparasite infestation in disturbed habitats, suggesting that proximity to forest edges can lead to decreasing ectoparasite prevalences in *Microcebus* spp. Such effects cannot be excluded here, as a comparison group from a larger continuous forest fragment is missing.

The population density of *M. griseorufus* at the study site was found to be comparably high, counting 11.8 individuals per ha (in TNP, not calculated in degraded habitats (Bohr et al., 2011). At BMSR up to 6.1 individuals per ha are present (Rasoazanabary, 2011), and a density of 4.3 Individuals per ha was calculated in the Berenty Reserve (Génin, 2008). Thus the low species richness of ectoparasites of *M. griseorufus* in the Tsimanampetsotsa region is likely not density correlated.

The grooming behavior of mammals is another important aspect regarding measures of ectoparasite infestation. Grooming is a widely employed behavior of hosts to remove ectoparasites such as ticks, lice and fleas (Akinyi et al., 2013; Hawlena et al., 2007). Primates not only groom in purpose of ectoparasite reduction, but allogrooming is also an activity to communicate social relationships and support integration and bonding to conspecifics (Akinyi et al., 2013; Barrett and Henzi, 2001; Hutchins and Barash, 1976; Lewis, 2010).Unlike higher primates that groom manually, lemurs use their "tooth comb" to groom themselves or group members (Sauther et al., 2002). In consequence some body parts like the head the upper back are inaccessible for autogrooming. (Hutchins and Barash, 1976), meaning a disadvantageous effect of solitary life style. However, it is assumed that the social aspect of grooming in lemurs may be more important than its hygienic function (Lewis, 2010).

Sleeping ecology may as well have an impact on parasite infestation. Hence, sleeping in tree holes apparently increases infestation with mites, lice and ticks, and sleeping in groups promotes only infestation with lice (Hokan et al., 2017; Klein et al., 2018).

Exposure and susceptibility to parasites has often been found to be sex-biased (Habig and Archie, 2015) probably due to behavioral traits or differences in testosterone levels (Zuk and McKean, 1996). Male *Microcebus* spp. are less often engaged in sleeping associations than females (Génin, 2013), males are the dispersing sex in *M. griseorufus* (Genin, 2008) and dispersal has been connected to higher loads of ectoparasites (Van Vuren, 1996). Female *M. griseorufus* get their fur groomed more often than males (Rasoanzanabary, 2011) which is likely due to the very common female dominance in lemurs (Génin, 2013). Accordingly, males of group living *Lemur catta* host more mites, ticks and lice than females (Sauther et al., 2002; Sauther et al., 2006). On the other hand, independence of ectoparasite prevalence from sex in lemurs is rather the rule (Hokan et al., 2017; Klein et al., 2018; Springer et al., 2015), which corresponds to our record of a non-sex-biased louse infestation for *M. griseorufus* from the Tsimanampetsotsa littoral zone.

Pathogens detected among ectoparasites

Tick-borne rickettsioses are among the oldest known vector- borne diseases (Parola et al., 2005). However, *Rickettsia* species continue to be detected in or isolated from arthropods around the world, and several species are found to be pathogenic only long after their discovery (Parola et al., 2013). *Rickettsia africae* is widely distributed on the African continent and has been detected in at least 13 hard tick species (Halajian et al., 2018; Parola et al., 2013) and in one bird flea species (Sekeyová et al., 2012). *Rickettsia felis* has been associated with 12 flea species in continental Africa (Brown and Macaluso, 2016; Reif and Macaluso, 2009).

Each of the 13 species of ticks, fleas or lice were tested positive for *Rickettsia*, the majority of which resembling *R. africae* genetically (99% to 100% identity of ompB). Apart from *Pulex irritans* all ectoparasite species provided *R. africae* DNA. With regard to the hosts, it means that all five endemic mammals trapped during this study are exposed to *R. africae* through infestation by the arthropod host of the bacterium. That concerns 16 out of 102 *M. griseorufus*, constituting a potential threat to the health of the population. Still, it is unknown if the ectoparasites are capable of rickettsiae transmission and if *R. africae* is pathogenic to their hosts. However, there are examples of small mammals that are infected with rickettsiae (Schex et al., 2011).

Only in 2010, Hornok et al. reported the first finding of *Rickettsia* in lice of livestock. Yet, only human body lice were experimentally proved to not only acquire, but maintain and inoculate flea-borne and tick-borne rickettsiae (Houhamdi et al., 2003; Houhamdi and Raoult, 2006). High prevalence of *R. africae* beyond 50% seems to be common for *A. variegatum* ticks. (Ehounoud et al., 2016; Lorusso et al., 2013; Vanegas et al., 2018; Yssouf et al., 2014), but the *R. africae* prevalence in Malagasy *Amblyomma* ticks seems to be extraordinary high. *Amblyomma variegatum* ticks showing 100% prevalence are in line with previous studies in Madagascar (Ehlers et al., 2016; Keller et al., 2016). Also the results of *R. microplus* with 16% containing *R. africae* DNA outperforms the values reported from other countries

(Ehounoud et al., 2016; Ogo et al., 2012; Vanegas et al., 2018; Yssouf et al., 2014). It is therefore notable that human infections by spotted fever group rickettsiae are only anecdotally documented in Madagascar (Fournier et al., 2009). African tick bite fever caused by *R. africae* was second to malaria the most frequently identified illness among travelers returning from Sub-Saharan Africa in the last years (Parola et al., 2013).

The other rickettsiae found in this study derived from fleas and were 100% homologous to *R. felis* (one pool of *C. felis*.) or clustered together with Candidatus *R. senegalensis,* as well known to infect cat fleas (Mediannikov et al., 2015), and now in the present study detected in *X. cheopis*. *P. irritans* and *E. gallinacea*, which originated from houses, from trapped mice or collected from the floor, respectively. Only a single further study from Madagascar has found *R. felis*, also in *X. cheopis* and in *P. irritans* fleas (Rakotonanahary et al., 2017). Despite several reports implicating *R. felis* as a prevalent cause of fever worldwide (Angelakis et al., 2016), and despite the growing number of newly discovered rickettsiae infecting fleas (Brown and Macaluso, 2016; Nziza et al., 2019), systematic studies on the ecology and pathogenicity of these bacteria are lacking.

Borrelia theileri (Smith et al., 1978), a species causing bovine borreliosis vectored by *Rhipicephalus microplus*, which is well established over the whole country (Stachurski et al., 2013). In face of the presence of the potential vector, borrelioses of domestic and wildlife animals are not reported from Madagascar. In the course of this study only three tick individuals were found to include *Borrelia* DNA: *R. microplus* (n=2) and *H. simplex* (n=1). Unexpectedly, one of the *R. microplu*-derived *Borrelia* DNA sequences grouped together with the reptile associated cluster of the genus in the phylogenetic analysis. The other *Borrelia* DNA sample obtained from *R. microplus* was indeed similar to *R. theileri* 16S rDNA (99.7%). *B. theileri* seems to be a rare pathogen of *Rhipicephalus* spp., often infecting less than 1% of the ticks (McCoy et al., 2014; Yparraguirre et al., 2007). The nucleotide sequence derived from the *Haemaphysalis* DNA sample showed highest similarity to *B. duttoni*, the causative agent of endemic relapsing fever in human, indicating that tenrecs and their ticks could be reservoir for the pathogen, however the suitable vector for humans, *Ornithodoros moubata,* is absent from the survey region. However, further *Borrelia* genes of the *Hemaphysalis* sample have to be sequenced to confirm the species.

The currently reported presence of *Bartonella* sp. in Madagascar is limited to fruit bats and their ectoparasites (Brook et al., 2015), to rats and *S. fonquerniei* fleas (Brook et al., 2017), to *H. lemuris* (Lado et al., 2018) and in both human head (Eremeeva et al., 2019) and body lice (Sangaré et al., 2014). Ticks (Angelakis et al., 2010a; 2010b) and fleas (Fernandez-Gonzalez et al., 2016; Sackal et al., 2008) seem to be commonly infected by bartonellae elsewhere, but not at our study site. Whereas *Bartonella* DNA was detected in pools of two different louse species: A potentially new *Bartonella* variant, closely related to other bartonellae infecting ruminants, was detected in the cattle louse *H. quadripertusus*. According to the *rpoB* phylogeny the same strain had also formerly been detected in *H. quadripertusus* from Israel (Gutiérrez et al., 2014). *Bartonella* DNA isolated from *L. verruculosus*, presumably from a novel *Bartonella* strain as well, revealed closest relationship (90%) to both pathogenic *B. henselae* and *B. quintana*.

Conclusion

Considering the vulnerability of the ecosystem and the present threat to its fauna it is important to assess a potential risk for animals due to disease. These baseline data may help to provide insights into the role of vector-borne diseases as possible threat to wildlife, and into their zoonotic potential (Leendertz et al. 2006). Human mediated alteration of the environment, through agricultural practices, livestock regime and introduction of foreign species are considered as main factors that drive disease emergence (Gortazar et al., 2014).

I characterized a broad spectrum of ectoparasites and a broad range of vector-borne bacteria were occuring in that particular rural region of southwest Madagascar, where humans contact to livestock and wildlife is high. Rickettsiae show the largest distribution within the ectoparasite fauna of the region, whereas borreliae and bartonellae were both found to be restricted to only two ectoparasite species each. Most ectoparasites likely do not have the potential for cross-species infestation, and hence disease transmission due to their assumed host specificity. However, pathogenic bacteria have been detected in ectoparasites which are known to feed on humans (ticks and fleas), most concerning the molecular detection of *Y. pestis* in the main vector *X. cheopis*.

The species richness of the ectoparasite community among small mammals in the Tsimanampetsotsa region is comparably low, most likely due to unfavorable conditions such as aridity and high temperature. Most species of ectoparasites that tend to cope with the dry climate in the coastal zone adjacent to the Tsimanampetsotsa National Park, outside of the shelters provided by human settlements, belong to the order of Phthiraptera. This is probably because lice develop entirely on the host lacking off-host phases during their lifecycle that are the most vulnerable stages in fleas and ticks. Three endemic tick species which are most likely host specific, dwelling in their hosts burrows or shelters, have been found to defy the climatic conditions. Only one flea species (*E. gallinacea*) is abundant outside of villages, precariously, it is introduced and obviously managed the leap from human settlements to forest habitats, now established as a parasite of the endangered *G. grandidieri.*

Microcebus griseorufus revealed the highest louse prevalence inside the least degraded habitat, contrary to the common assumption that the prevalence of ectoparasites increases in degraded areas. It should be taken into consideration that ecto-parasitism may not be just an indicator for degradation, but ectoparasites themselves could be adversely affected by habitat alteration, diminishing their ecosystem service by regulation of host populations. The negative factors of degradation may carry more weight compared to a weakened resilience of hosts that would redound to the ectoparasites' advantage.

Some impetus for future research emerges from this dissertation. As this study indicates the prevalence of a broad range of vector-borne bacteria in wide spectrum of ectoparasites it is therefore urging to an investigation of according bacterial infections and reservoir function of the hosts. This study is the cornerstone for subsequent investigations which address the epidemiological relevance of the vector-borne pathogens detected. Seroprevalence studies for past infections can reveal disease burdens in the local human population, livestock and the endemic fauna. Additionally, the arthropod's vector competence has to be proven. Bacterial cultures and inoculation experiments could address these questions. There is also a need to analyze the ecology of the ticks presented here, especially off-host, to learn more about their distribution range and life cycles. Direct search for ticks in hosts nests and burrows, forest understory, and in the grasslands of the plateau is indicated.

The results presented here, should raise awareness among residents to combat the thriving of rats inside villages more consistently. The study also provides evidence that shepherds should be sensitized to establish a tick management to protect their animals. A strict containment of the risk factors would protect animal as well as human health.

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Julian Ehlers