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AN EVALUATION OF THE ORAL MICROBIOME AND POTENTIAL ZOOONES OF TWO UBIQUITOUS SOUTH AFRICAN STREPSIRRHINES, *OTOLEMUR CRASSICAUDATUS* AND *GALAGO MOHOLI*.

BY

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B.A. University of North Dakota, 2011

A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirement for the degree of Master of Arts. Department of Anthropology 2014
This thesis entitled: 
An Evaluation of the Oral Microbiome and Potential Zoonoses of Two Ubiquitous 
South African Strepsirrhines, *Otolemur crassicaudatus* and *Galago moholi*. 
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Find that both the content and the form meet acceptable presentation standards 
Of scholarly work in the above mentioned discipline.
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An Evaluation of the Oral Microbiome and Potential Zoonoses of Two Ubiquitous South African Strepsirrhines, *Otolemur crassicaudatus* and *Galago moholi*.

Thesis directed by Professor Michelle L. Sauther

As *Otolemur crassicaudatus* and *Galago moholi* are ubiquitous throughout part of South Africa they are thought to be at little conservation risk. However, this has resulted in a situation where these species are seldom studied. This thesis addresses this lack of knowledge about these two species by investigating the oral microbiome of each for potential zoonoses. This is an important undertaking due to the widespread nature of these species which could provide an avenue for potential zoonoses to spread.

In order to assess the oral microbiome, two genetic sequencing methods were utilized. Real-time PCR was utilized to search for mycobacterial contributors to the oral microbiome such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Next-gen Illumina sequencing of the 16s region was chosen as an effective method to get an overall quantification of the amount of different bacterial contributors. Through these two methods major bacterial contributors of each tested oral microbiome were isolated and those with potential for causing illness in humans or domestic animals were identified.

The results illustrated a potential core microbiome for all *Otolemur crassicaudatus* consisting of three separate bacterial contributors; *Mannheimia caviae*, *Porphyromonas catonia*, and *Gemella cunicula*. While all tested *O. crassicaudatus* exhibited these three contributors the rest of the oral microbiome was made of different contributors in varying amounts. The qPCR (real-time PCR) for mycobacteria (tuberculosis and leprosy) yielded negative results despite several potential initial amplifications.

Despite qPCR findings several potentially pathogenic bacterial strains were discovered through next-gen 16s sequencing. These included *Fusobacterium naviforme*, *Streptococcus bovis*, and *Gemella cunicula*; all of which are known to cause disease in human hosts. A number of other bacterial strains with both human and non-human health risks were discovered in wild *O. crassicaudatus* as well as in captive sampled *Galago moholi*. These findings thus form the basis for several future avenues of potential research to assess any real zoonotic risks from these galagos to the broader human and animal populations of southern Africa.
Dedication

This thesis is dedicated to my family without whose encouragement, inspiration, and support this work would not have been possible.
Acknowledgements

Growing up in a house with a medical professional and a former serology technician as parents instilled in me a curiosity for health and how the body reacts to diseases. Through continued study into epidemiology and immunology I have furthered my understanding of how populations as well as the human body react in the presence of both known and novel pathogens. What I could not understand however is how new diseases emerge and how we can seemingly do so little to prevent the spread in the early stages of a truly novel pathogen.

In order to better fill in the gaps in my knowledge, as well as some of those of the medical field, I have decided to focus my studies on the emergence of diseases from non-human primate sources and how these can contribute to epidemics of known and novel pathogens. Whether the great epidemics from history or the recurring pandemic scares which happen every few years I have become fascinated by how some diseases manage to appear from nowhere and can ravage an entire population while others stay endemic to a group without causing any significant long lasting damage to the population as a whole. Diseases which we are not accustomed to can have devastating effects as we have little to no inherent resistance to them, an example being the introduction of smallpox to the New World and the massive death toll this caused in the native populations. To this end I have decided to study how new diseases emerge, where they come from, and how to alleviate and prevent new pandemics from occurring in the modern era.

For their theoretical, instructional, and material contributions to the research behind this thesis I would like to thank Drs. Michelle Sauther, Frank Cuozzo, Krista Fish, Adrian Tordiffe, Anne Stone, Helene Brettschneider, Steven Strinden, and Ms. Genevieve Hausman. Without their expertise and guidance this work would not have been possible. Dr. Ian Gaigher, director of Lajuma Research Centre deserves special acknowledgement for his creation of the research area and his support of our project within it. I would also like to thank the staff of the National Zoological Gardens of South Africa, especially Dr. Antoinette Kotze, Mark Howitt and Lee Smith for their assistance in locating, sampling, and sequencing samples from Galago moholi. Dr. Adrian Tordiffe also deserves another acknowledgement for his help in the field as well as his assistance in procuring laboratory contacts and lab assistance and instruction. In recognition of their financial support allowing for the acquisition of supplies, materials, transport, and other ancillary needs I also thank the CU Museum, the University of North Dakota, Colorado College, and the Beverly Sears grant committee. Finally, for their support and encouragement I would finally like to thank the employees of Lowe’s store 0220, Derek Mihm, and James Millette who helped me through the frustrations of delving into personally unknown territories in order to author this work.
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Chapter 1: Introduction

Zoonoses and Emerging Pathogens

Zoonoses (diseases with the ability to transfer between human and non-human hosts) are a growing area of research and interest in today’s epidemiological world. It is well documented that one of the major causes of novel and emerging pathogenic infection is due to transmission from zoonotic sources (Wolfe et al., 1998). According to data released in 2013 by the Centers for Disease Control and Prevention approximately 75% of newly emerging pathogens are zoonotic in nature while at least 60% of known human pathogens have a zoonotic component (CDC, 2014b). However, what is largely unknown is where the next potential pandemic may emerge from, or when this may occur. Given the recent fear of pandemics associated with zoonotic transmissions, such as SARS, H1N1, or Avian Influenza, it has become increasingly important to investigate potential reservoirs of zoonotic infection for novel pathogenic involvement in addition to those which are already known to harm humans and livestock. These pathogens which spread from non-human animals; i.e. livestock, avians, and non-human primates, to humans are often poorly understood even when pathogens are identified, such as the previously mentioned SARS. This lack of understanding can lead to widespread panic and often culling of important economic and subsistence animal groups for fear of disease spread.

In order to understand why zoonoses play such a pivotal role in understanding epidemiology we can observe some of the most infamous pandemics in history and find at their core a non-human contributor or instigator, which caused them. The year 1918 yields one such example as the Spanish Flu, the first appearance of H1N1 a.k.a. the Swine Flu, hit the world becoming the first modern pandemic (Tumpey, 2005). This disease had multiple components resulting in its wide effects including the huge migrations of human populations during World War 1 and the growth of agriculture to feed a larger
industrialized world population. The lack of understanding of how humans and animals spread diseases led to the death of as much as 5% of the world’s population in a single year (Tumpey, 2005).

Another example from history of the risks from a lack of understanding of the role animals may play in epidemiology lies in the Black Plague of the 14th century. It is now widely accepted that the Black Plague was caused by rats carrying vectors for the bubonic and pneumonic plagues into port cities in Europe and then into the countryside (Ziegler, 2013). The declaration by Pope Gregory IX in 1232 that cats were creatures of the devil led to a systematic prejudice and extermination of cats throughout Europe. With this destruction of the natural predator, rats were allowed to propagate nearly unchecked, priming the pump for the introduction of plague rats from the Far East. This human intervention for religious reasons then indirectly resulted in the death of approximately one third of the population of Europe, an event that may not have happened if the ability of rats to carry disease had been understood. Lack of understanding of the zoonotic risks in European society is therefore the catalyst of one of the worst pandemics in recorded history and is one of the reasons for a better understanding of non-human primates, a group known to carry diseases fatal to humans (Hahn, 2011; Rouquet, 2005).

One such disease and a modern example of the importance of understanding and monitoring known zoonotic contributors to human epidemics is currently playing out in western Africa with the Liberian outbreak of Ebola which has since spread to Guinea, Sierra Leone, Senegal, and Nigeria (CDC, 2014a). A source at the CDC contacted through the CDC telephone information line confirmed that the likely cause of the current outbreak was a non-human primate (Feldmann, 2003; Personal Correspondence, 2014) as all of the sporadic outbreaks since the discovery of Ebola in 1976 have originated from a non-human primate source, primarily due to the consumption of infected bushmeat. While bats are the natural host species for Ebola the hypothesized main method of transmission has involved infection of a primate before consumption or butchering although the exact method of
transmission is unknown (Groseth, 2007). The method in which these epidemic catalysts themselves become infected is still unknown and is a further reason investigations into non-human primate epidemiology must be undertaken.

This outbreak of Ebola has repercussions far beyond simply viewing the death toll or rate of spread. The spread of disease can cause fundamental changes in international politics and human nature, such as the fact that the Liberian National Defense Minister, Brownie Samukai, says that this single outbreak has placed the collapse of the country’s existence in sight (Nichols, 2014). There are also historical precedents of diseases causing massive social upheavals. The Black Plague caused the switch to the feudal state as we know it today (Daileader, 2014). Similarly, the Plague of Justinian in 541CE ravaged the Byzantine Empire causing a collapse in population to the point they had to ask for military assistance even 500 years later to retake lands lost due to population shrinkage (what became the First Crusade) (Harl, 2014). It is abundantly clear that disease is a major force in the social and political world far beyond mere concepts of health as we think of.

**Non-Human Primates as Important Disease Reservoirs for Zoonotic Diseases**

Non-human primates (NHP) are reservoirs of zoonoses for a couple of important reasons. Firstly, they are taxonomically and genetically close to humans and as such their parasites and pathogens are more likely to jump the species boundary than are those from other taxonomic groups (Guerrera, 2003). Secondly, increasing human populations and anthropogenic pressures are pushing humans and non-human primates into closer and more frequent proximity creating more opportunities for pathogenic transfer (Engel, 2012; Chapman, 2005). Lastly, primates are frequently relocated (De Thoisy, 2001), kept in zoos, homes, and research centers which drastically increases the amount of contact researchers, conservationists, zoo workers, and pet owners have with primates which are often taken from wild
populations (Guerrera, 2003). This is often done as a result of habitat loss which is also one of the major causes of zoonotic transfer and emergence of novel pathogens in its own right (De Thoisy, 2001).

There is strong evidence of novel pathogens making the leap from non-human primates to humans and some of our most prevalent, as well as most feared diseases stem from a primate source. For example, the widespread parasite *Plasmodium vivax* which causes most cases of malaria world-wide (Mendis, 2001) was recently shown to have stemmed from a macaque parasite (Escalante, 2005). Another example of important zoonotic pathogen transfer to humans from non-human primates is one of the most feared diseases in the world, Ebola (Engel, 2012).

These examples of ways that zoonotic pathogen transfer can occur along with some potential diseases which can transfer underline the importance of studying primates as potential vectors as well as reservoirs for both known and novel pathogens.

**Microbiomes**

One important way to understand what pathogens a given species harbors is through investigation of their microbiomes; the bacterial, viral, and parasitic inhabitants of a given area of a host. The most commonly studied areas for microbiome studies are the intestinal tract, the oral cavity, and the vaginal cavity (Li, 2013). All of these areas harbor large amounts of bacteria, many of which are commensal and symbiotic with the host such as the microbiota of the human gut (Hooper, 2001).

Studies have previously been conducted on both *Homo* and *Pan* salivary and oral microbiomes in order to understand bacterial diversity and individual health (Li, 2013). However, use of the oral microbiome as a predictor of zoonoses is a new and potentially important research approach.

**The Importance of Africa for Understanding Zoonotic Diseases Linked to Primates.**
With non-human primates being such a prominent reservoir, continental Africa can be viewed as a hotbed of both identified and potential threats to not only human but also domesticated and wild fauna. As primate pathogens are genetically similar to those that infect *Homo sapiens* (Wolfe et al., 1998), i.e. genetic similarities between the various strains of primate *Plasmodium* and those that infect humans, wild populations of primates living within proximity to human and livestock habitations are especially relevant for disease monitoring.

The southern tip of Africa is important as a breeding ground for potential human infection from numerous vectors. Malaria, West Nile, and Dengue are commonplace, especially in locales such as northern South Africa, Zimbabwe, and Mozambique, as are a myriad of more mundane pathogens (Health Information, 2014). In South Africa alone, during 2012, there were 530,000 diagnosed cases of tuberculosis, a disease reaching pandemic standards on most continents; excluding Australia and Antarctica. Of these 530,000 confirmed cases 31,000 proved fatal, while almost 120,000 of these were new incidences of human contraction of tuberculosis contracted during 2012 (WHO, 2013). In comparison, due to medical and infrastructure advancements, the United States experiences a tuberculosis rate of less than 5 cases per 100,000 individuals (WHO, 2013). These numbers mean that at least 857 out of every 100,000 people in South Africa suffer tuberculosis infections each year, a rate 171 times higher than that of the United States, and that this number is on the rise.

Likewise, countries neighboring the South African province of Limpopo exhibited elevated rates of tuberculosis infection; 433 out of 100,000 individuals in Zimbabwe and 343 per 100,000 individuals in Botswana (WHO, 2013). This demonstrates a regional elevation of tuberculosis risk in the southern regions of Africa and as such increases the importance of identifying those species which might be contributing to such epidemic levels of infection.
Tuberculosis is not the only disease making resurgence in the regions in and around South Africa; leprosy, a disease of great historical importance, which is genetically related to tuberculosis, is also beginning to return to many less developed countries around the world (WHO, 2014). In South Africa during 2012 48 out of every 100,000 people were diagnosed with some form of leprosy (WHO, 2014). Likewise, in bordering Botswana 15 out of every 100,000 inhabitants were afflicted (WHO, 2014).

**Study Species**

These epidemic demographic numbers call for a greater understanding of all the contributing factors to the spread and success of tuberculosis and leprosy in Africa as opposed to more traditionally developed countries. As noted above, one such factor to be considered is the reservoir capabilities of endemic primates, which can alter the disease landscape considerably both by harboring known human pathogens and by presenting a pathway by which novel pathogens may enter human populations. To this end, a study was conducted on two geographically-diverse species of strepsirrhine primates from the Republic of South Africa; *Otolemur crassicaudatus* and *Galago moholi* to better gauge their potential for regional disease spread and emergence. In addition, this study also assessed whether galagos might be important for maintaining an endemic level of a given disease (e.g. sustaining a critical size or density of a host population below which a disease cannot exist, Deredec and Courchamp, 2003).

*Figure 1:* Two *Galago moholi* kept as pets in a domestic setting. Photo courtesy of Mr. Patrick Evans.
*Otolemur crassicaudatus*. The greater, or thick-tailed, Galago, which averages a weight of approximately 1,384g (Nekaris and Bearder, 2011), feeds primarily on gums and fruits and has been shown to complement its diet with insects (Nekaris and Bearder, 2011). This species is characterized by “monkey-like” quadrupedal locomotion with some hopping evident (Nekaris and Bearder, 2011). *O. crassicaudatus* is known to have between 2 and 3 offspring a year (Nekaris and Bearder, 2011). This species is found primarily in more rural areas where they have been spotted as small family groups (Clark, 1984), primarily in larger vegetation such as tall trees routinely surrounded by lower scrub foliage and acacia trees (Nekaris and Bearder, 2011). Most observations on this species have been conducted in riverine forests where *O. crassicaudatus* was primarily located in the lower strata of vegetation (Nekaris and Bearder, 2011). Our study site however is more akin to cloud forest but the activity pattern was largely the same as previous studies indicated.

*Galago moholi*. This species is, a small galagid with an average weight of 200g (Bearder, 1987; Nekaris and Bearder, 2011), and while it is found sympatrically at Lajuma with *O. crassicaudatus* it is more often allopatric and is ubiquitous throughout northern South Africa as well as Botswana, Malawi, Namibia, and Tanzania (Bearder, 1987; Nekaris and Bearder, 2011). Their preferred habitat is low acacia scrub and young growth trees (Nekaris and Bearder, 2011). The diet of these smaller species is primarily insectivorous though gum feeding has been noted as well (Nekari and Bearders, 2011). The smaller *G. moholi* has a maximum of 2 offspring a year (Nekaris, and Bearder 2011). This species is also found in urban environments as well; anecdotal evidence from a doctoral student at the National Zoological Gardens in Pretoria, Rutger Spies, has seen lesser galagos living in trees within the zoological gardens as well as in trees, on telephone poles, and on power lines within urban environments outside of his residence (Spies, personal correspondence, 2013). *G. moholi*, in contrast to *O. crassicaudatus* is characterized by specialized leaping and bipedal hopping behaviors (Bearder, 1987; Nekaris and Bearder, 2011). Prior studies of this species have focused on their behaviors in acacia woodlands and
savanna biomes (Nekaris, 2011), precisely the type of environment G. moholi was observed in at Lajuma. Average range size for G. moholi is between 10 and 20 hectares with ranges of both males and females overlapping (Bearder, 1987; Nekaris and Bearder, 2011).

As is the case with other exotic pets (Associated Press, 2006), these galago species are of special importance to this study as they can often be found kept as wild-caught pets (Figure 1) and as such pose a much higher risk of disease transmission than many primates, which simply live near human populations. This increase in risk is due to the increased opportunity for fomites, which are physical contact vectors, and airborne vectors to affect humans due to prolonged and close contact with these animals, often within their own houses (Stehr-Green, 1987).

Rationale for This Study

To understand the potential effects galagos could have on the epidemiological landscape of southern Africa I employed genetic methods for identifying and quantifying the bacteria found within the oral cavity of galagos as well as ran specific genetic tests for barcode sequences of specific diseases. The oral cavity was selected both for ease of sampling as it is minimally invasive as well as for the propensity of different bacteria to colonize the oral cavity due to the combination of both hard tissue surfaces and mucosal surfaces being present (Zaura, 2009). In order to identify the presence or absence of specific bacterial contributors, i.e. tuberculosis or leprosy, a technique pioneered in bacterial diagnostics in the 1990s was used, that of qPCR or real-time PCR (Telenti, 1993). Also, a newer technique known as Illumina sequencing (Cheeseman, 1994), developed by Drs. Balasubramanian and Klenerman of Cambridge was employed to ascertain the variety of bacterial contributors within the sample population to better understand the risks or both known and unknown bacterial strains which can be found in wild galagos (Tedersoo, 2010).
The reasoning behind selecting qPCR for identification of *Mycobacterium tuberculosis* and *Mycobacterium leprae* lies in the level of specificity that is possible and the ability to perform these tests even after storage of genetic materials in the field. As Polymerase Chain Reactions (PCR) takes a specific segment of a genome and multiples it to a detectable amount if even a single copy of that target strand is present it allows for even significantly degraded DNA to be analyzed. Due to the high level of precision PCR also allows for a single type of genetic material to be looked for even if the sample represents a mixture of many contributors providing the PCR primer set is specific enough (Tewari, 2011).

In contrast to the specificity of real-time PCR is the use of 16s Illumina sequencing, a next-gen sequencing technique which allows an investigator to parse out how many contributors are in a mixed sample as well as what genus, and sometimes species, these contributors come from. The ability to identify samples in a bacterial mixture is only limited by the fact that most bacterium have never been sequenced or added to genetic libraries and as such are difficult to pinpoint from a sample of many contributors. This technique also allows much higher resolution results compared to traditional PCR methods giving greater insight into the contents of each sample (Tewari, 2011). Utilizing next-gen sequencing methods also allows for identification of bacterial contributors which ordinarily do not appear in cultures are do not survive long enough outside of the host to be cultured, ie. streptococcal strains (Quinn, 2011).
Chapter 2: Methods

Project Framework

The framework for this study was developed in conjunction with an investigation into the behavioral ecology, health, and biology of greater and lesser galagos within South Africa headed by Drs. Michelle Sauther, Frank Cuozzo and Krista Fish. The project follows the basic protocols created during their many years of work on *Lemur catta*, another strepsirrhine primate and was carried out under the University of North Dakota’s Animal Welfare Assurance (IACUC # A3917-01). This research aims to assess the ecological niches occupied by the two sympatric species of galagos as well as their population density, health, and preferred environment of each species. My contribution to the project was to assess the oral microbiome of the galagos in order to understand the type and diversity of bacterial groups found within isolated populations of galagos as well as to identify any known pathogens which may be active in the population. Drs. Sauther and Fish conducted nocturnal surveys to ascertain behavioral traits and preferred environments as well as measuring biometrics and collecting ectoparasites from captured samples. Dr. Cuozzo assessed the dental health and tooth wear and made dental casts to understand the dental ecology of the study species (Cuozzo and Sauther, 2012).

Anesthesia and welfare monitoring as well as humoral sampling was performed by Dr. Adrian Tordiffe of the National Zoological Gardens of the RSA.

Collaboration on qPCR investigation of mycobacterium was done with Dr. Anne Stone of Arizona State University and her students, notably Genevieve Hausman. Dr. Stone supplied sampling materials for gathering salivary samples from captured individuals as well as providing laboratory training and material to myself in order to investigate the presence or absence of both *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Dr. Stone studies the impact and occurrence of these two pathogenic
mycobacterium in New World Monkeys, armadillos, and prehistoric Amerindian skeletal populations (Klaus, 2010; Stone, 2009) and agreed to facilitate my investigation in order to begin her investigations on the African continent.

Another vital collaborator was Dr. Antoinette Kotze and the research branch of the Zoological Gardens of South Africa. Through Dr. Kotze’s guidance and involvement I was put in contact with Drs. Desire Dalton and Helene Bretteschneider who assisted with developing and implementing both preliminary Sanger sequencing protocols and next-gen Illumina sequencing with the Agricultural Research Center. The laboratory at the NZG through this collaboration gave me genetic sequencing training and provided storage for one set of my collected samples for further investigation through work with Dr. Brettschneider. This invaluable assistance allowed the results presented here which will also result in submission of an article within the coming year.

**Environmental and Ecological Assay**

Sampling and observation of wild samples occurred at Lajuma Private Reserve in Limpopo province (S 23° 02.285, E 29° 26.441), Republic of South Africa, during the months of June and July, 2013. This site consists of a mosaic of habitats that includes marshland, thicket and riverine forest (Willems, 2007). The neighboring parcels however have been altered by humans, with a ranch along one border and a tourist lodge in a neighboring parcel. Also, one area of the reserve was formerly used as fruit plantations before being obtained by the reserve. This allowed us the opportunity to investigate both relatively undisturbed and highly disturbed areas for galago activity and potential behavior alterations.

To ascertain which environments were inhabited by both thick-tailed and lesser galagos census walks were conducted shortly after dusk. Three separate transects through the reserve were chosen by Drs. Fish and Sauther which followed primary roads and paths with each transect being surveyed
multiple times throughout the study period. Locations that were visually confirmed by Drs. Fish and Sauther to have galagos were then visited during the day in order to characterize the habitat around the area of sighting. A rectangular area 10 meters by 20 meters was marked off with the tree where the galago was spotted in the center. All trees within this square were measured for trunk width, distance from sighting tree, and height in order to better understand the preferred environment of the species sighted (Fish, 2014).

**Specimen Capture and Sample Collection for Wild Samples**

Lajuma Private Reserve was selected for its reasonable geographic isolation and the low incidence of fragmented habitat within the reserve. During this period, walks were conducted along main reserve thoroughfares to assess areas of likely galago habituation. Six Havahart™ traps, three small and three medium, and three custom-made Chardonette traps (Figure 2) were then zip-tied into trees in those areas suspected of galago activity. Traps were moved to new locations after several nights of no captures, or after repeated captures of previously sampled individuals (identified via sub-dermal radio frequency identification (RFID) chips). Each trap was baited at dusk using a mixture of banana and honey set on a piece of bark on the trigger plate in order to attract individuals. During those nights when trapping occurred, each trap was checked hourly until either 9pm or until a maximum of 3 novel individuals were obtained for sampling, whichever came first. Each captured individual was scanned for an injected RFID chip before transfer to an overnight container so as to limit the occurrence of repeated sampling of the same individuals. Those individuals to be sampled were then transferred from the Havahart™ traps to domestic pet travel carriers or modified
plastic storage tubs by the project’s wildlife veterinarian, Dr. Adrian Tordiffe, and I, by means of a pillow-case equipped with a drawstring closure. These individuals were then kept in their carriers within our laboratory building until the following morning, when enough light was available to allow sampling.

Prior to collecting data from the captured individuals, they were anesthetized using a subdermal injection using Zoletil™ at a dose of between 4.8mg/kg and 7.5mg/kg, with a mean dose of 7.2mg/kg, and a single-use, commercially available insulin syringe until unconscious. The amount of anesthetic was determined as a percentage of overall individual weight, as calculated by Dr. Tordiffe. Vital signs and safety were monitored and assured by Dr. Tordiffe and his staff to prevent any harm or undue stress to the subjects. Subsequent anesthesia was administered using inhaled Isoflurane™ in doses between 2-4% mixtures with air as prescribed in Larsen et al. (Larsen, 2011). Once sedated, subdermal radio frequency identification (RFID) chips were inserted between the scapulae and confirmed with a portable RFID reader. The last four characters of each RFID tag were then assigned on all data collections as the individuals’ sample numbers. The sex and age grade of each individual was also noted. Age grades were defined as either subadult, being sexually undeveloped or adult, being sexually mature. Data collection then began with basic biometrics that included length, weight, skinfold measurements, presence of ectoparasites by the use of a tape measure, digital scale, and digital calipers. Dental ecology and health was assessed using a scale of wear that was developed and tested on Lemur catta (Cuozzo and Sauther, 2006; Millette, 2009) and dental casts were made. The oral microbiome was sampled using two distinct kits, Thermo Scientific AssayAssure™, and a kit designed specifically for collection of salivary mycobacterial samples as conceived by Dr. Stone of ASU (Appendix 1). Blood was drawn for analysis of nutritional and humoral health. Finally, urine samples were collected through manual manipulation of the bladder and fecal samples were taken opportunistically from each individual’s container as available to assess parasites. A total of 23 individual Otolemur crassicaudatus were sampled at Lajuma with one unintended repeat bringing the total samples collected from Lajuma to 24.
After testing was completed, subjects were held in individual carriers until approximately an hour prior to dusk. After this time subjects were transported to their respective locations of initial capture and were released. Researchers remained on site until subjects were out of view, to ensure there were no evident complications from the capture and testing process.

**Humoral Analysis**

Blood and urine samples were analyzed on site by our wildlife veterinarian for preliminary findings on nutritional and health status. These analyses included differential blood counts, specific gravity, and pH tests to determine each individual’s health status.

**Sampling of Captive *Galago moholi*.**

In addition to the wild caught *O. crassicaudatus* three captive *G. moholi* were also sampled. This was accomplished through the assistance of Mark Howitt of the National Zoological Gardens of RSA and head of the Mokopane Biodiversity Conservation Centre. Mr. Howitt was able to bring us to a local woman’s home who was accommodating a number of galagos for rehabilitation. The galagos were kept in a moderately sized cage, approximately 12 feet square with a divider through the center separating the *G. moholi* cage into halves for a solitary individual on one side and the other half of the cage being occupied by a pair. These individuals were sampled without sedation by inserting swabs into their mouths and allowing them to chew and lick the swab for a short amount of time. The local woman assisted by catching and securing the galagos manually while I quickly swabbed the oral cavity of each captive *G. moholi*. The galagos were quite habituated to humans, were often handled by this individual and this did not appear to cause any distress.

**Salivary DNA Storage**
The swabs containing the oral microbiome samples were placed directly into buffering solutions, sealed, and labeled with the appropriate specimen identification number. These samples were stored at ambient temperature of no less than 10°C and no more than 30°C until returned to the wildlife laboratory at the National Zoological Gardens Pretoria, Republic of South Africa. At this point samples undergoing active DNA extraction and amplification were stored at 4°C indefinitely at these facilities. Those samples which were being reserved for later testing were kept at ambient temperature until return to the United States at which point they were placed in a commercial freezer until tested.

16s Illumina sequencing

16s DNA Extraction and Detection. At the NZG I extracted and purified DNA from the collected samples under the guidance of Andreas, a NZG intern and lab technician. This study was conducted by extracting genomic DNA from salivary samples using the ZR Genomic DNA™ Tissue MiniPrep kit from Zymo Research according to specifications laid down by the manufacturer. Elution of the DNA was conducted using 50 uL of elution buffer and was stored at 4°C until further analysis. Each sample was screened for the presence of bacteria with a universal primer set which amplified a 1400bp region of the 16sRNA gene (Frank, 2008). In order to avoid cross-contamination pre- and post-PCR processes were conducted in separate laboratories; DNA extraction, PCR, and sequencing laboratories respectively, and all PCR reactants were prepared in a DNA-free hood, after sterilization by means of 10 minute exposure to a UV light source. A final control against cross contamination involved inclusion of a DNA-free control during PCR testing.

Gel electrophoresis was conducted in order to test success of the PCR amplification. Samples were considered successfully amplified if the band amplified to expected size. All positively amplified samples were then purified using the Zymo PCR Product Purification Kit and were subsequently cycle sequences using BigDye v. 3.1 terminator cycle-sequencing kit from Perkim Elmer, Foster City. U.S.A.
Sequencing was run on an ABI 3130 sequencer and resulting chromatograms were edited and visualized using the Chromas program embedded in Mega 5 (Tamura, 2011) before searching for matching nucleotide sequences using BLAST (www.ncbi.nlm.nih.gov/blast) in hopes of identifying probable bacterial species within each sample. Several sequences of high probability in each sample were selected for further investigation at a later date.

Genetic analysis of each of the 24 samples yielded amplicons of sufficient length and were then sequenced for species identification of the bacterial contributors. The resulting chromatograms were largely mixed however which suggests multiple bacterial contributions within each sample. Use of BLAST on small segments of relatively clear signal also yielded inconclusive results as to which bacterial species were present and were thereby removed from further analysis. Due to the inconclusive nature of the results, next generation sequences methods were then used to distinguish between the multiple contributing bacterial DNA samples allowing results which could not be obtained through normal sequencing and culturing methods (Ronaghi, 2007; Tedersoo, 2010; Tewari, 2011).

Ten of the successful samples were chosen by myself and Dr. Brettschneider for further next-gen sequencing offsite at the Agricultural Research Center (ARC) in Johannesburg, South Africa. These samples were then run through a BLAST genomic identification program to identify each different bacterial contributor (Voelkerding, 2009). This process would yield more accurate species level results than previously used Sanger sequencing allowing differentiation between even very similar bacterial strains by creating a consensus sequence from large numbers of parallel sequences (Voelkerding, 2009). Another benefit to choosing this method for further study is that it allows us to differentiate with a great deal of certainty between even morphologically similar bacteria such as Enterococcus cecorum and Streptococcus bovis (Tewari, 2011).
The top eight contributions, by relative volume of the total amount of reads for the microbiome (# of reads for strain/total # of reads), were chosen to provide a percentage of the total bacterial load they represent and those which were present in all of the eight *Otolemur crassicaudatus* samples were analyzed using the Mann-Whitney U-tests, due to the low n value achieved, to investigate potential sex differences. Significance was set at $p \leq 0.05$.

**Tuberculosis and Leprosy**

**Leprosy and Tuberculosis DNA Extraction** DNA extraction for testing the presence of either Tuberculoid or Leproric DNA markers occurred during February, 2014, in the laboratory of Dr. Anne Stone at Arizona State University in Tempe. The DNA extraction and subsequent qPCR testing methodology, which were previously utilized on several South American primates and American nine-banded armadillos, *Dasypus novemcinctus*, were devised by Dr. Anne Stone and Ms. Genevieve Hausman, MA; both of Arizona State University before being provided to me for use. Extraction and qPCR analysis were performed by myself at the Primate Lab, Stone Lab, and the General Lab at Arizona State University during this period.

DNA from swab samples was stored at 4°C until extraction in order to preserve DNA integrity between time of collection and time of extraction. Prior to extraction of DNA each sample was incubated at 80°C for a period of one hour in order to kill any remaining harmfully viable bacteria which may have been present and to prevent contamination of the laboratory facility. Upon completion of the incubation period 25uL of Proteinase K (10mg/mL) was added in order to lyse cells within the collected sample. The samples were then mixed with a pipette and placed into a rotisserie incubator at a temperature between 55°C and 65°C for a period of one hour to catalyze the lysing process.

Extraction was performed using a Phenol-Chloroform method. 250uL of phenol and 250uL of chloroform: isoamyl alcohol, were added to each sample and mixed via inversion of the sample containers. Samples were then centrifuged at 12,000rpm for two minutes at room temperature before
transferring the top aqueous layer to a phase-lock gel tube. The remaining interphase, organic phase, and collection swab were placed in a biohazard container for disposal. 250ul more phenol and 250ul more chloroform: isoamyl alcohol was added to each phase-lock tube and once again samples were mixed through inversion. Samples were then once again centrifuged at 12,000rpm for two minutes at room temperature. 500uL of chloroform: isoamyl alcohol was added to each sample and mixed via inversion. One more round of centrifuging at 12,000rpm for 10 minutes was conducted in order to separate the desired DNA from organic and chemical adherents. Finally, the top aqueous phase of each phase-lock gel tube was transferred to a new tube and the phase-lock gel tube was placed into biohazard disposal.

The extracted DNA was then separated from the extraction chemicals through an alcohol precipitation regimen. 3M of NaOAc was added to each sample in a ratio equaling 1/10 of the total aqueous solution acquired from the extraction phase. Chilled isopropyl alcohol was also added to each solution in a ratio of 6/10 of the total extracted solution. After addition of the two compounds each sample was incubated at 4°C for a period of 1 hour. Without thawing the samples were then centrifuged at 13,000rpm for 15 minutes at 4°C. After centrifuging the supernatant was decanted from each sample in order to remove the majority of the extraction chemicals. After decanting 1mL of chilled 70% ethanol was added and inverted to mix the samples. Samples were then centrifuged again at 13,000rpm for 15 minutes at 4°C. After centrifuging the supernatant was once again decanted and each sample pellet was dried in a vacu-fuge for 15 minutes at 30°C.

DNA was finally re-suspended once cleared of the extraction chemicals in 50uL of low TE buffer with a pH of 8.0. Once the buffer was added samples were placed on a shaker tray at 100rpm for at least 24 hours to ensure complete re-suspension of extracted DNA. Each sample was also nano-dropped in order to ascertain whether extraction protocols were successfully completed (Appendix 2). After nano-
dropping was completed samples were stored at 4°C until prepared for qPCR via creation of 5ng/μL working solutions through combining extracted DNA and sterile water to make 100μL of working solution per original sample.

**qPCR Testing.** Protocols for mycobacterium qPCR tests were provided by Dr. Stone and Ms. Hausmann, both of Arizona State University. Testing was conducted in the Stone Lab, Primate Lab, and General Lab at ASU under the supervision the direct supervision of Ms. Hausmann.

Four separate qPCR tests were run, two for Tuberculosis DNA and two for leprotic DNA. Each pair consisted of one primer set designed to identify multivariate regions and one primer set designed to identify a stable barcode sequence for either Tuberculosis or Leprosy respectively. The four primer sets consisted of a forward primer, a reverse primer, and a fluorescent qPCR probe sequence in order to detect amplification through fluorescence (Appendix 3).

Optimized concentrations for each primer were previously identified by Dr. Anne Stone and Genevieve Hausman, MA, and where thus added for best resolution of any potential amplifications. Each qPCR reagent set was therefore made of varying concentrations of forward primers, reverse primers, probe primer, and Taq Master Mix (Appendix 4).

After creation of the Master Mix 18μl was added to each well which would hold a sample or control in a 16x24 PCR plate. Standards were included in pairs from a concentration of 5e⁻¹ to 5e⁻⁵ ng/μL in order to provide robusticity and accuracy to standard measurements. A pair of non-template controls consisting of low-TE buffer were also included to test for and control against contamination of the Master Mix. Samples were included in triplicate to allow for detection of false positives or possible contamination. Each control, NTC, and sample was added at a volume of 2μL bringing the total volume of each utilized well to 20μL.
Once controls, NTCs, and samples were added a sterile adhesive sheet was affixed to the surface of the qPCR plate preventing leaks and external contamination during the remainder of the testing phase. Due to the photosensitive nature of the probes each sealed and prepared PCR plate was wrapped in tinfoil to preserve the reagents until such time as the plate was placed into the qPCR machine.

Bubbles were removed from wells by smacking each qPCR plate upside down against a counter several times and then centrifuging at 3000rpm for 10 minutes, this process was repeated as needed until no bubbles remained in the samples to interfere with readings.

Actual testing of each sample was conducted using an automated qPCR machine attached to a computer running a SDS software suite. A new file was created for each individual test to prevent confusion in interpreting results. Each tray was then run through a full set of qPCR cycles while the equipment monitored for increases in fluorescent probe activity. There were three overall stages of cycles, the first stage consisted of one cycle of two minutes at 50°C, the second stage was a single cycle of 10 minutes at 95°C, and the third stage consisted of 50 cycles between 95°C for 15 seconds and 60°C for one minute. At the completion of all stages and cycles a computer readout was generated and interpreted using SDS software to check for successful controls as well as possible amplifications of sample materials.

In the event of suspected amplifications, or potentially inhibited amplifications, another qPCR plate was set up with only standards, NTCs, and the amplified samples. Each of the potential amplifications was retested at a 10x dilution of the working solution to alleviate any mitigating effects of extraction reagents which might still be present in the samples. Another session was conducted using the qPCR machine and results were again interpreted using SDS software to view successful standards and success or failure of retested sample amplification.
Chapter 3: Results

16s

Preliminary sequencing of genetic samples yielded inconclusive specific results and signaled a large variation in bacterial donor DNA evident in the samples tested. When run through BLAST several high probability bacterial contributors were identified for further investigation with better targeted primer sets. The most likely contributors in the samples tested were *Vibrio cholera* (Cholera), *Mannheimia haemolytica* (Shipping Fever), and *Haemophilus influenza* (Pfeiffer’s bacillus). Samples have been sent to an external lab in South Africa for advanced sequencing and metagenomics of a number of bacterial contributors as well as identified individual pathogens. This next-gen sequencing was used to identify the overall number of bacterial species in each sample as well as accurate identification of identifiable microbial contributors.

qPCR

Initial qPCR testing of each of the two leprosy genome markers failed due to the inclusion of incorrect control samples for the standards (Appendix 5, Figures 1 and 2). This resulted in an inability to accurately assess the amplifications, or lack thereof, of the test samples. Proper control samples were identified and each leprosy identification qPCR was rerun with successful standards (Appendix 5, Figures 3 and 4) and no amplifications were detected in any of the tested samples.

The two tests for tuberculosis genomes returned successful standards (Appendix 5, Figures 5 and 6) and a possibly inhibited amplification of a single galago salivary sample in each genomic marker, rpoB2 and IS6110. The qPCR for rpoB2 returned a single potentially inhibited amplification in one of the three redundant 85EF wells (Appendix 5, Figure 7). The test for IS6110 returned possibly inhibited amplifications in two of the three redundant 9BC7 wells (Appendix 5, Figure 8).
Retesting of the samples using the inhibited amplification protocols outlined earlier yielded another set of successful standards (Appendix 5, Figures 9 and 10) and no amplifications in either 85EF, for the rpoB2 sequence, or of 9BC7, for the IS6110 sequence.

**Humoral and Fecal Analysis**

Analysis of urine, fecal matter, and biometric measurements of each of the samples yielded no significant aberrations in physical health. Two individuals, 3B9F and 21A9, had very low specific gravity for their urine samples however this is likely due to hydration rather than physical malady. Likewise, fecal samples exhibited low incidence of parasite activity, primarily *Entamoeba sp.* and *Giardia sp.*, and as such is likely a negligible contributor to overall galago health due to the scarcity found.

**Next-Gen Illumina sequencing**

Using next-generation sequencing techniques the number of bacterial contributors in ten randomly selected oral microbiomes were investigated. Of these ten random samples 8 were wild caught *Otolemur crassicaudatus* and 2 were captive *Galago moholi*. The overall number of bacterial contributors, both identifiable and non-identifiable, were parsed from the samples as well as the top 8 bacterial contributors by volume in each of the samples (Table 1).

The frequency of each identified genus and species was also identified so as to better understand the demographics of the oral microbiome (Appendix 6, Figures 1-20). Using these frequencies a series of statistical tests were run to investigate the possibility of sex differences in the total amount of bacterial contributors and in the frequency of involvement of shared contributors such as *Mannheimia caviae*, *Gemella cunicula*, and *Porphyromonas catoniae* (Table 2). No significant sex difference in any tested bacterial contributor was identified ($p \leq 0.05$).
As can be seen in Table 1 as well as in the frequency charts (Appendix 6, Figures 11-20) most separate species that could be distinguished have not been identified clinically. This resulted in a large amount of the results being due to unknown and unidentified bacterial contributors. Those bacterial strains that were identified were investigated for possible pathogenic involvement of both humans and livestock (Table 3).

There were also differences in the types and distribution of potentially pathogenic bacterial strains between the wild caught *Otolemur crassicaudatus* and the captive sampled *Galago moholi*. While both species shared commensal populations of both *Mannheimia caviae* and *Gemella cunicula* the rest of the oral microbiome was quite different in terms of both identified bacterial species and proportion of unclassified contributors (Figures 3 and 4). The variety of bacterial contributors and their relative proportions within the oral microbiomes of the two sexes were also evaluated for evidence of significant differences (Figures 5 and 6). No significant (P<0.05) differences were found.
Figure 3: Pie chart showing the relative abundance of each species of bacterial contributor out of 2,669,265 useable sequencing reads for *Otolemur crassicaudatus*. 

**Proportional Abundance (# of Reads) of Bacterial Species in the Oral Microbiome of 8 *Otolemur crassicaudatus***
Figure 4: Pie chart showing the relative abundance of each species of bacterial contributor out of 959,028 useable sequencing reads for *Galago moholi*.
Figure 5: Pie chart showing the relative abundance of each species of bacterial contributor out of 1,255,301 useable sequencing reads for female Otolemur crassicaudatus.
Figure 6: Pie chart showing the relative abundance of each species of bacterial contributor out of 1,413,964 useable sequencing reads for male *Otolemur crassicaudatus*. 
Table 1: Number of distinct bacterial contributors to each specimen’s oral microbiome as well as top 8 contributors by volume (Number of reads identified/total number of reads).

<table>
<thead>
<tr>
<th>Specimen</th>
<th># of Contributors</th>
<th>Highest Contributor</th>
<th>2nd Highest</th>
<th>3rd Highest</th>
<th>4th Highest</th>
<th>5th Highest</th>
<th>6th Highest</th>
<th>7th Highest</th>
<th>8th Highest</th>
</tr>
</thead>
<tbody>
<tr>
<td>C53F</td>
<td>703</td>
<td>Unclassified at Species Level</td>
<td>Mannheimia caviae</td>
<td>Gemella cunicula</td>
<td>Moraxella caviae</td>
<td>Porphyromonas catoniae</td>
<td>Streptococcus bovis</td>
<td>Butyrivibrio proteoclasticus</td>
<td>Rothia aeria</td>
</tr>
<tr>
<td>3C15</td>
<td>790</td>
<td>Unclassified at Species Level</td>
<td>Mannheimia caviae</td>
<td>Porphyromonas catoniae</td>
<td>Gemella cunicula</td>
<td>Streptococcus bovis</td>
<td>Butyrivibrio proteoclasticus</td>
<td>Actinobacillus pleuranumoniae</td>
<td>Sneathia sanguinegens</td>
</tr>
<tr>
<td>5FB7</td>
<td>682</td>
<td>Unclassified at Species Level</td>
<td>Mannheimia caviae</td>
<td>Gemella cunicula</td>
<td>Porphyromonas catoniae</td>
<td>Streptococcus bovis</td>
<td>Laeotropia mirabilis</td>
<td>Moraxella caviae</td>
<td>Planococcus maritimus</td>
</tr>
<tr>
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<td>652</td>
<td>Unclassified at Species Level</td>
<td>Mannheimia caviae</td>
<td>Gemella cunicula</td>
<td>Porphyromonas catoniae</td>
<td>Fusobacterium naviforme</td>
<td>Sneathia sanguinegens</td>
<td>Ureaplasma gallerale</td>
<td>Streptococcus peronis</td>
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<td>Mannheimia caviae</td>
<td>Gemella cunicula</td>
<td>Porphyromonas catoniae</td>
<td>Ureaplasma gallerale</td>
<td>Butyrivibrio proteoclasticus</td>
<td>Streptococcus peronis</td>
<td>Fusibacterium naviforme</td>
</tr>
<tr>
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<td>Mannheimia caviae</td>
<td>Butyrivibrio proteoclasticus</td>
<td>Porphyromonas catoniae</td>
<td>Gemella cunicula</td>
<td>Fusobacterium naviforme</td>
<td>Aggregatibacter aphrophilus</td>
<td>Leptotrichia goodfellowi</td>
</tr>
<tr>
<td>5010</td>
<td>792</td>
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<td>Mannheimia caviae</td>
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<td>Butyrivibrio proteoclasticus</td>
<td>Gemella cunicula</td>
<td>Fusobacterium naviforme</td>
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<td>Aggregatibacter aphrophilus</td>
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<td>Butyrivibrio proteoclasticus</td>
<td>Streptococcus bovis</td>
<td>Moraxella caviae</td>
<td>Laeotropia mirabilis</td>
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<td>Captive 1</td>
<td>887</td>
<td>Unclassified at Species Level</td>
<td>Mannheimia caviae</td>
<td>Neisseria lactamica</td>
<td>Aggregatibacter aphrophilus</td>
<td>Streptococcus sanguinis</td>
<td>Rothia aeria</td>
<td>Streptococcus peronis</td>
<td>Gemella cunicula</td>
</tr>
<tr>
<td>Captive 3</td>
<td>883</td>
<td>Unclassified at Species Level</td>
<td>Mannheimia caviae</td>
<td>Gemella cunicula</td>
<td>Streptococcus peronis</td>
<td>Corynebacterium durum</td>
<td>Aggregatibacter aphrophilus</td>
<td>Veillonella dispar</td>
<td>Avibacterium avium</td>
</tr>
</tbody>
</table>
Table 2: Values and results of Mann-Whitney U-tests run to investigate potential differences in bacterial load between sex classifications of specimens tested.

<table>
<thead>
<tr>
<th># of Contributors</th>
<th>Sex</th>
<th>Age</th>
<th>% of reads</th>
<th>% of reads</th>
<th>% of reads</th>
<th>% of reads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mannheimia</td>
<td>Gemella</td>
<td>Porphyromonas</td>
<td>Unknown</td>
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<tr>
<td>684</td>
<td>Female</td>
<td>Sub Adult</td>
<td>17.28</td>
<td>2.54</td>
<td>2.87</td>
<td>57.81</td>
</tr>
<tr>
<td>792</td>
<td>Female</td>
<td>Adult</td>
<td>8.66</td>
<td>3.33</td>
<td>4.87</td>
<td>66.68</td>
</tr>
<tr>
<td>901</td>
<td>Male</td>
<td>Sub Adult</td>
<td>13.24</td>
<td>7.02</td>
<td>10.58</td>
<td>42.56</td>
</tr>
<tr>
<td>790</td>
<td>Male</td>
<td>Adult</td>
<td>23.21</td>
<td>3.6</td>
<td>11.53</td>
<td>45.98</td>
</tr>
<tr>
<td>652</td>
<td>Male</td>
<td>Adult</td>
<td>12.8</td>
<td>3.21</td>
<td>2.63</td>
<td>67.35</td>
</tr>
<tr>
<td>682</td>
<td>Female</td>
<td>Sub Adult</td>
<td>14.82</td>
<td>6.01</td>
<td>2.69</td>
<td>64.46</td>
</tr>
<tr>
<td>554</td>
<td>Female</td>
<td>Adult</td>
<td>29.29</td>
<td>10.09</td>
<td>5.26</td>
<td>41.19</td>
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<tr>
<td>703</td>
<td>Male</td>
<td>Adult</td>
<td>16.86</td>
<td>5.55</td>
<td>2.87</td>
<td>59.62</td>
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</table>

<table>
<thead>
<tr>
<th>Contributors x Sex</th>
<th>Mannheimia x Sex</th>
<th>Gemella x Sex</th>
<th>Porphyromonas x Sex</th>
<th>Unknown x Sex</th>
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</thead>
<tbody>
<tr>
<td>U-Value = 11.0</td>
<td>U-value = 9.0</td>
<td>U-value = 8</td>
<td>U-value = 8</td>
<td>U-Value = 11.0</td>
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<tr>
<td>p = 0.386476</td>
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<td>p = 1</td>
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<td>z = 0.866025</td>
<td>z = 0.288675</td>
<td>z = 0</td>
<td>z = 0.433013</td>
<td>z = 0.866025</td>
</tr>
</tbody>
</table>
Table 3: Identified bacterial pathogens and their known effects.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Human Pathogenic Involvement</th>
<th>Livestock Pathogenic Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gemella cunicula</em></td>
<td>Endocarditis, Meningitis, Arthritis, and Pneumonia</td>
<td>No Known Pathogenic Effects</td>
</tr>
<tr>
<td><em>Streptococcus bovis</em></td>
<td>Colorectal Cancer, Endocarditis, Meningitis, and Neonatal Septicemia</td>
<td>Ruminal Acidosis and Feedlot Bloat</td>
</tr>
<tr>
<td><em>Rothia aeria</em></td>
<td>Septic Arthritis and Endocarditis</td>
<td>No Known Pathogenic Effects</td>
</tr>
<tr>
<td><em>Actinobacillus pleuropneumoniae</em></td>
<td>No Known Pathogenic Effects</td>
<td>Pneumonia</td>
</tr>
<tr>
<td><em>Sneathia sanguinegens</em></td>
<td>Septic Arthritis</td>
<td>No Known Pathogenic Effects</td>
</tr>
<tr>
<td><em>Fusobacterium naviforme</em></td>
<td>Lemierre Syndrome and Generalized Inflammation</td>
<td>No Known Pathogenic Effects</td>
</tr>
<tr>
<td><em>Streptococcus peroris</em></td>
<td>Endocarditis</td>
<td>No Known Pathogenic Effects</td>
</tr>
<tr>
<td><em>Aggregatibacter aphrophilus</em></td>
<td>Endocarditis</td>
<td>No Known Pathogenic Effects</td>
</tr>
<tr>
<td><em>Avibacterium avium</em></td>
<td>No Known Pathogenic Effects</td>
<td>Sinusitis and Pneumonia</td>
</tr>
<tr>
<td><em>Mannheimia caviae</em></td>
<td>No Known Pathogenic Effects</td>
<td>Conjunctivitis</td>
</tr>
</tbody>
</table>
Chapter 4: Discussion

Results indicate that at Lajuma there is no discernible difference in the bacterial variety or the percentage of each load made up of specific bacterium between the sexes. This is largely to be expected as *Otolemur crassicaudatus* is fairly social (Clark, 1984) and as such allows many chances for the microbiome to spread between individuals creating a group norm.

The investigation of mycobacterium loads yielded negative results across the board for both leprosy and tuberculosis. While this means that this particular population of sampled individuals are not harboring these mycobacterium it does not preclude the possibility that they may be able to do so elsewhere, i.e. a more populated area. Thus, we cannot discount the possibility of these strepsirrhines acting as an endemic reservoir for these two pathogens elsewhere (Deredec and Courchamp, 2003). In contrast to the narrow focus of the qPCR results an interesting, though statistically insignificant, result was the variety of bacteria that are linked to communicable diseases found in *Galago moholi* who were only successfully sampled in small numbers from captive populations found through Illumina sequencing. Even though the sample size was rather small there were still several potentially pathogenic strains evident. Those populations held in captivity were expected to exhibit greater species of bacteria linked to zoonotic disease, due to persistent proximity to humans and domesticated animals. This prediction was upheld as they contained *Aggregatibacter aphrophilus*, *Rothia aeria*, *Streptococcus peroris*, *Gemella cunicula*, and *Avibacterium avium*. These four bacterial species are known to cause disease in humans and livestock ranging from opportunistic meningitis to septic arthritis in humans and sinusitis to pneumonia in livestock.

The wild samples from *O. crassicaudatus* oral microbiomes were largely dominated by three bacterial species, which were found in all samples. *Mannheimia caviae*, *Gemella cunicula*, and *Porphyromonas catoniae* are commensal in all samples and make up a majority of the identifiable
microbiome. This contrasts with previous studies of human populations where it was found that there are no single species with an overwhelming presence in the human oral microbiome (Lazarevic, 2010; Zaura, 2009). These three bacterial contributors can then potentially be considered the “universal core” (Lazarevic, 2010), meaning all *O.crassicaudatus* will exhibit these bacteria in addition to their individually diverse loads. While these results only pertain to Lajuma further study can elucidate whether this proves to be true in other regions as well.

Humans could also be at risk from galago-borne diseases, especially in the context of keeping galagos as pets or using them as bush meat. In South Africa as humans encroach on their natural environments, galagos are often pushed out of their forests due to real estate development which leads to them being injured on roads, bitten by domestic pets and even direct encounters between humans and these primates (Brulliard, 2010). In addition, among the poorer residents of Johannesburg, they are often caught to sell as pets or used in traditional medicine or as bushmeat (Brulliard, 2010). The investigation of the oral microbiome revealed many bacterial strains that are opportunistically pathogenic to humans, primarily those which cause endocarditis, a potentially fatal inflammation of cardiac tissues due to injury or severe illness (Mayo Clinic, 2014). As was shown previously in Table 3 the sampled oral microbiomes contain a variety of opportunistic pathogens that can afflict humans. This can pose large health risks to individuals who are already immune-compromised with diseases such as HIV, which is rampant in South Africa with approximately 6.3 million infected individuals (UNAIDS, 2014). Likewise, the young and the elderly are at increased risk of these opportunistic pathogens especially with the minimal access to health care in most areas. Also, the presence of drug-resistant tuberculosis strains throughout South Africa, when coupled with the high incidence of HIV, poses dire health consequences (Gandhi, 2006).
Another key result is the apparent interplay in bacterial ecology between *O. crassicaudatus* and domestic bovids in plots neighboring Lajuma. One potential bacterial microbiome contributor, discovered through 16s Illumina sequencing in 40% of tested samples, was *Streptococcus bovis*, a disease which causes neonatal septicemia and meningitis in humans and which is known to cause feedlot bloat in cattle (Russell, 1985). *Otolemur crassicaudatus* at Lajuma, by harboring this bacteria, has the potential ability to transmit this bacteria and thus the disease to livestock, as transmission of such a disease is extremely unlikely to be a one-way street (Goldberg, 2008). Also, as previously observed, livestock are the most common intermediary between non-human primate and human disease transmission (Goldberg, 2008). However, it remains to be seen how severe this interplay is and what epidemiological risks can be linked to it. Likewise, the exact vector of this transmission is unknown and whether these diseases can be transmitted to livestock as well as contracted from them requires further study to prove. Importantly, the results indicate that strepsirrhines are able to harbor the same strains (sub-species) of pathogenic bacteria and as such can be assumed to have the ability to distribute these species to their normal hosts. This has large conservation implications given that if a clear link between galagos and livestock illness can be proved then galagos may face active extermination rather than neutral tolerance.

Likewise, the discovery of *Avibacterium avium* in one of the captive samples is cause for concern as this pathogen, normally commensal in birds, has been shown to cause sinusitis and pneumonia in calves. There is also concern for swine kept in proximity to *O. crassicaudatus* populations, as the discovery of *Actinobacillus pleuropneumoniae* in 10% of the investigated samples is known to cause pneumonia in domesticated swine. This pathogenic discovery outlines a potentially dangerous interaction due to the varied diet of *O. crassicaudatus* overlapping that of potential forage eaten by swine such as fruit in areas where pigs are a prevalent livestock product.
Another avenue of potential transmission is through the preponderance of house pets (dogs and cats) at Lajuma Private Reserve. This has not been studied as a potential vector both of diseases to humans and to the primates within the reserve. Domestic cats are of special concern as they exist at Lajuma in relatively high numbers and have been witnessed far from human habitation points in the brush where galagos have been observed (Personal Observation). Cats pose a threat of spreading diseases to galagos as they are a primary carrier of *Bartonellosis*, or cat-scratch disease. This zoonotic pathogen has seen a rise in prevalence in developing and industrialized countries and poses a growing risk to humans and animals (Breitschwerdt, 2010) This means that even unsuccessful predation attempts on galagos at Lajuma could result in contraction of this disease with symptoms including swollen lymph nodes, swollen joints, and lethargy (Kahn, 2010) as well as rheumatic symptoms and long-term fever (Jacobs, 1998; Kahn, 2010); a suite of traits which can result in a higher likelihood of predation. In turn the predator would then be exposed to this disease causing wide-ranging repercussions to the entire ecosystem. It is precisely because of situations like this that the bacterial load of galagos should be better understood, not only for their health but for that of their ecosystem as well. As such it is not just passive transfer that is a risk to human health but active predation by human commensals such as domestic cats, and this could yield another means of domestic animals acquiring novel pathogens. With the outbreak of Ebola in Africa this is of concern as fomites, vectors involving touch, could spread to animals fur and then to humans through simple touch (Mani, 2009). This would create a direct line of transmission between humans and galagos, or vice versa, with only a single intermediary in the form of the household animal (cat or dog) to mediate the potential transfer.

The results from Lajuma point to a wide variety of bacteria associated with known communicable diseases. However, most of these pathogenic bacterial species are opportunistic rather than aggressive pathogens. However, this does not mean galagos are not carriers and catalysts of outbreaks in the rest of the region however as these results also show an ability for these strepsirrhines
to act as latent reservoirs for pathogens increasing the number of individuals available for a latent endemic disease threshold. Lajuma was chosen due to its isolated nature and as such, was hoped to have few human maladies, but the majority of South Africa has undergone extensive anthropogenic change, which could radically alter the vector capabilities of primates living there. As previously stated the neighboring parcels are occupied by more commercial concerns such as a ranch, which also increase the risk of pathogen transfer between galagos and livestock in this area.

The large number of previously unidentified bacterial strains is also an important finding. This indicates that despite almost two decades of research into cataloguing and sequencing genomes there is still a great deal of work to be done. It is because of this gap in our knowledge that we are unable to say if those strains are potentially hazardous or benign in this population.

This project was important not only to provide a better understanding the health status of a specific population of galagos but also for establishing the protocols and methodologies for expanding the study of galago’s oral microbiomes to new geographic areas. This expansion would allow a greater assessment of how regional and anthropogenic differences impact disease ecology and the risk primates can present to their human neighbors. This study also offers important baseline data to provide a greater understanding of how susceptible non-human primates can prove to be to human pathogens and vice-versa, something of great concern to conservationists, wildlife biologists, and zoo health officials (De Thoisy, 2001; Engel, 2012)

While all species of Galago are listed as Least Concern by the IUCN the results shown earlier indicate that biological and epidemiological knowledge of these species is needed regardless of conservation status. In fact, as these species are of Least Concern their biological state could result in a much larger impact than those primates which have been studied epidemiologically due to their conservation concerns, i.e. *Pan troglodytes*. As galagos have a much larger geographic range and are
more likely to have contact with human populations, both rural and urban, understanding their biology is actually of greater importance than those of the geographically isolated “Great Apes”.

In addition, genetic surveys such as the one conducted here can prove invaluable for detecting bacteria even if an individual or animal is currently undergoing anti-microbial treatments (Wolfe et al., 1998). This can affect the way veterinarians and wildlife caretakers look after their patients, as a normal culture test is unlikely to show positive results if tested while antibiotics are in use. Genetic assaying can however reveal bacterial involvement in diseases even when antimicrobials are in use, allowing for better and more accurate treatment for animals. Understanding what bacterium naturally occur in galagos will also allow health care professionals differentiate what is an aberration as opposed to what is normal and healthy for an individual (Zaura, 2009).
Chapter 5: Ongoing Research and Conclusions

Future research avenues are currently being planned with regards to both more intensive investigation of the salivary microbiome as well as into regional variations in galago disease ecology throughout South Africa, Botswana, and Namibia. As these study genera are so prevalent throughout the southern regions of Africa, further investigation is required to fully understand potential health and conservation implications, not only of the galagos themselves but also of their human and domesticated neighbors. As such, sampling in several areas throughout these countries is proposed, in locations including game reserves, agricultural zones, urban areas and shelters/zoos. With a wider cross section of both inhabited areas and a larger study population the ability of galagos to act as reservoirs of disease can be better assessed. This would allow not only greater understanding of the contributions of galagos to a potential disease but also allows for truer studies of epidemiology and disease spread as medical intervention is not required as is the case when studying epidemics in human populations (Wolfe et al., 1998).

In addition to the regional variations in galago microbiome inhabitants I plan to further investigate the number of differentiated bacterial contributors in each sample beyond simply identifying a few major contributors. By quantifying the number of bacterial contributors per region, age, or sex of the subject through next-generation Illumina sequencing my goal is to identify factors most likely to contribute to the emergence of novel pathogens through understanding which combination of variables can yield the highest variation of undocumented bacterial DNA.

A tertiary line of proposed research would attempt to identify the rate at which habitat disturbances alter the salivary microbiome of South African galagos. This is of importance to understanding epidemic trends and threats from these endemic primates as alteration or destruction of
habitat has shown a correlation with accelerated transmission of bacterium between primates, livestock, and humans (Goldberg, 2008), a major factor in the contraction of intestinal parasites (Salzer, 2007), as well as expanding the potential pool of hosts for a given disease (Wolfe et al., 1998). This area of research would also prove important to conservation concerns as such documented pathogen exchange shows no directional bias in infectivity between humans, their domesticates, and wild primates (Goldberg, 2008).

Finally, a survey of livestock salivary biomes would be beneficial to undertake as livestock show evidence of being the most common intermediary between non-human primate and human disease transmission (Goldberg, 2008).

In conclusion, as I have shown, the potential for zoonoses from strepsirrhine sources in Africa is a subject that warrants further study. While these results show the capability of galagos to harbor pathogen strains, the extent to which they act as vectors for these diseases is still unknown. This study has provided baseline knowledge for bacterial loads in relatively intact environments, but now more work is needed to fully understand how this differs in various environments throughout the region and what vector effects these animals may cause.
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http://etheses.dur.ac.uk/2559/


Appendix 1: Custom Cell-Lysis Buffer Ingredients

- 50mM Tris pH 8.0
- 50mM EDTA
- 50mM Sucrose
- 100mM NaCl
- 1% SDS
### Appendix 2: Nanodrop Concentrations and Working Dilution Volumes

<table>
<thead>
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<th>H2O Volume (µL)</th>
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Appendix 3: Forward primers, Reverse primers, and Fluorescent probes used for mycobacterial qPCR

rp082 Primer (forward)
(Mycobacterium tuberculosis)
Optimized Final qPCR Concentration = 50nM
48 TaqMan Custom Primer
Name: rp082-F
Sequence: (5') CAA CGT CGA GGT GCT ATC G (3')
Dry
Desalted
10,000 pmoles

rp082 Primer (reverse)
(Mycobacterium tuberculosis)
Optimized Final qPCR Concentration = 300nM
48 TaqMan Custom Primer
Name: rp082-R
Sequence: (5') CTC CAG GTC CTC GTC CTC A (3')
Dry
Desalted
10,000 pmoles

rp08 Probe
(Mycobacterium tuberculosis)
Optimized Final qPCR Concentration = 300nM
48 TaqMan MGB Probe
Name: rp08 Probe
Sequence: (5') 6FAM - TCG CCG CAC CAC TAC CGT A - MGBNFQ (3')
1 x TE 100uM
HPLC
6,000 pmoles

Is6110 Primer (forward)
(Mycobacterium leprae single-copy gene)
Optimized Final qPCR Concentration = 50nM
48 TaqMan Custom Primer
Name: IS6110-F
Sequence: (5') GGG TAG CAG ACC TCA CCT ATG TG (3')
Dry
Desalted
10,000 pmoles

Is6110 Primer (reverse)
(Mycobacterium leprae single-copy gene)
Optimized Final qPCR Concentration = 300nM
48 TaqMan Custom Primer
Name: IS6110-R
Sequence: (5') CGG TGA CAA AGG CCA CGT A (3')
Dry
Desalted
10,000 pmoles

Is6110 Probe
(Mycobacterium leprae single-copy gene)
Optimized Final qPCR Concentration = 300nM
48 TaqMan MGB Probe
Name: IS6110 Probe
Sequence: (5') 6FAM - ACC TGG GCA GGG TT - MGBNFQ (3')
1 x TE 100uM
HPLC
6,000 pmoles

Rlep Primer (forward)
(Mycobacterium leprae multi-copy gene)
Optimized Final qPCR Concentration = 250nM
48 TaqMan Custom Primer
Name: F-RLEP
Sequence: (5') GCA GTA TCG TGT TAG TGA A (3')
1 x TE 20uM
Desalted
10,000 pmoles

Rlep Primer (reverse)
(Mycobacterium leprae multi-copy gene)
Optimized Final qPCR Concentration = 250nM
48 TaqMan Custom Primer
Name: R-RLEP
Sequence: (5') CGC TAG AAG GTT GCC GTA TG (3')
1 x TE 20uM
Desalted
10,000 pmoles

Rlep Probe
(Mycobacterium leprae multi-copy gene)
Optimized Final qPCR Concentration = 200nM
48 TaqMan MGB Probe
Name: rlep probe
Sequence: (5') 6FAM TCG ATG ATC CGG CGG TCG MGBNFQ (3')
1 x TE 100uM
HPLC
6,000 pmoles

85B Primer (forward)
(Mycobacterium leprae single-copy gene)
Optimized Final qPCR Concentration = 300nM
48 TaqMan Custom Primer
Name: 85B-qPCR-F
Sequence: (5') GTG GTC GGC CTC TCG AT (3')
1 x TE 20uM
Desalted
10,000 pmoles

85B Primer (reverse)
(Mycobacterium leprae single-copy gene)
Optimized Final qPCR Concentration = 50nM
48 TaqMan Custom Primer
Name: 85B-qPCR-R
Sequence: (5') CGA GCC AGC ATA GAT GAA CTG ATC (3')
1 x TE 20uM
Desalted
10,000 pmoles

85B Probe
(Mycobacterium leprae single-copy gene)
Optimized Final qPCR Concentration = 300nM
48 TaqMan MGB Probe
Name: 85B-qPCR-Probe
Sequence: (5') 6FAM - CTC GGC CCT AAT ACT - MGBNFQ (3')
1 x TE 100uM
HPLC
6,000 pmoles
**Appendix 4**: Optimized qPCR Master Mix ratios per reaction (uL)

<table>
<thead>
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<th>Gene</th>
<th>Universal Master Mix</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
<th>Rat Serum Albumin*</th>
<th>H₂O</th>
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<td>– 0.25</td>
<td>– 0.04</td>
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</table>

*10mg/mL
Appendix 5: qPCR amplification SDS standards and results for mycobacteria

Figure 1: Failed 85B qPCR Standards
Figure 2: Failed rlep qPCR Standards
Figure 3: Successful 85B qPCR Standards
Figure 4: Successful rlep qPCR Standards
Figure 5: Successful rpop2 qPCR Standards and Controls
Figure 6: Successful IS6110 qPCR Standards and Controls
Figure 7: Possible single replicant amplification of sample 85EF for Tuberculosis genetic sequence rpoB2
Figure 8: Possible double replicant amplification of sample 9BC7 for Tuberculosis genetic sequence IS6110
Figure 9: Successful rpoB2 standards and controls for re-test of 85EF
Figure 10: Successful IS6110 standards and controls for re-test of 9BC7
Appendix 6: 16s bacterial contributor ratios by volume
(# of bacterial reads/total microbial reads)

Top Genus Classification Results

- Gemella: 5.79%
- Moraxella: 4.22%
- Mannheimia: 16.86%
- Other: 20.93%
- Unclassified: 52.20%

Figure 1: Sample C53F identified bacterial genera by relative volume
Figure 2: Sample 3C15 identified bacterial genera by relative volume
Figure 3: Sample 5F87 identified bacterial genera by relative volume.
Figure 4: Sample 58A3 identified bacterial genera by relative volume.
Figure 5: Sample 98C7 identified bacterial genera by relative volume.
Figure 6: Sample 2979 identified bacterial genera by relative volume.
Figure 7: Sample 5010 identified bacterial genera by relative volume.
Figure 8: Sample 8123 identified bacterial genera by relative volume.
Figure 9: Sample Captive 1 identified bacterial genera by relative volume.
Figure 10: Sample Captive 3 identified bacterial genera by relative volume.
Figure 11: Sample C53F identified bacterial species by relative volume.
Figure 12: Sample 3C15 identified bacterial species by relative volume.
Figure 13: Sample 5F87 identified bacterial species by relative volume.
Figure 14: Sample 58A3 identified bacterial species by relative volume.
Figure 15: Sample 98C7 identified bacterial species by relative volume.
Figure 16: Sample 2979 identified bacterial species by relative volume.
Figure 17: Sample 5010 identified bacterial species by relative volume.

Top Species Classification Results for 5010

- Unclassified: 74%
- Porphyromonas catoniae: 5%
- Mannheimia caviae: 10%
- Butyrivibrio proteoclasticus: 5%
- Other: 6%
Figure 18: Sample 8123 identified bacterial species by relative volume.
**Figure 19:** Sample Captive 1 identified bacterial species by relative volume.
**Figure 20:** Sample Captive 3 identified bacterial species by relative volume.