A Noninvasive Approach to Understanding Adaptation, Crop Raiding Behavior, and the Fecal Microbiota of the African Elephant

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By

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The undersigned, appointed by the dean of the Graduate School,

have examined the Dissertation entitled

A NONINVASIVE APPROACH TO UNDERSTANDING ADAPTATION, CROP RAIDING BEHAVIOR, AND THE FECAL MICROBIOTA OF THE AFRICAN ELEPHANT

Presented by Tabitha Marie Finch

A candidate for the degree of

Doctor of Philosophy

And hereby certify that, in their opinion, it is worthy of acceptance.

Lori S. Eggert

J. Chris Pires

Gavin Conant

Reginald Cocroft

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CHAPTER 1:
INTRODUCTION

The African savanna elephant (*Loxodonta africana*), the world's largest land mammal, is internationally recognized as a species of conservation concern. African forest (*L. cyclotis*) and savanna elephants are both keystone species that help to shape their habitat by influencing canopy cover (Dublin, Sinclair et al. 1990), dispersing seeds (Blake, Deem et al. 2009), and affecting species distributions (Pringle 2008). Thus they play a vital role in the makeup of the ecosystems they inhabit. In addition, the elephant has great economic value in the tourism industry (Brown Jr 1993) and unfortunately also on the black market for its prized ivory tusks, for which it has become a leading symbol in the fight to end the illegal wildlife trade.

Studies of elephants, however, are often politically charged and hotly debated both within the scientific community and in the political world. One such debate concerns the taxonomy of the extant African elephant, with some believing that there is evidence that they should be divided into two distinct species, the savanna elephant and the forest elephant, and others believing these are subspecies (*L. a. africana* and *L. a. cyclotis*). Due to the wet and humid conditions in the African forest zone, fossils are rare and not well preserved, thus making it difficult to accurately estimate divergence times of many taxa whose most recent common ancestor inhabited the forest zone. However, with recent advances in sequencing technology and the acquisition of whole

mitochondrial genomes for both the woolly mammoth (*Mammuthus primigenius*) (Rogaev, Moliaka et al. 2006) and the American mastodon (*Mammut americanum*) (Rohland, Malaspinas et al. 2007), studies on the relationships among the Elephantidae have been possible. Specifically, one group of researchers combined both mitochondrial and nuclear markers and estimated that the African forest and savanna elephant diverged approximately 5.5 million years ago (Roca, Georgiadis et al. 2001, Brandt, Ishida et al. 2012).

As compared to savanna elephants, forest elephants are significantly smaller, have longer, thinner and straighter tusks, more rounded ears, and a flatter forehead region (Martin 1991). Their diet consists of woody browse and fruit (Short 1981, Tangley 1997) whereas savanna elephants are generalist grazers/browsers that consume 60-95% of their forage as grasses (Codron, Codron et al. 2011). These sister species have adapted to their respective ecosystems by developing specialized ecological features. Because these differences are related to metabolic activity, in Chapter 1 I analyzed the protein-coding genes of the mitochondrial genome for signals of positive selection. These genes code for the machinery that makes up the oxidative phosphorylation pathway, which produces the majority of ATP needed for cellular processes. After identifying amino acid residues that may be under positive selection, I used homologous protein modelling to further assess the physicochemical effects these residue changes might have on the overall structure and function of the enzyme. This

study aimed to address the mechanism for genetic adaptations at the DNA sequence level in non-model species.

Human-elephant conflict is a prevalent issue spanning the geographic range of both Asian (*Elephas maximus*) and African elephants, and the most widespread form occurs when elephants raid agricultural fields, a behavior known as crop raiding (Sukumar 1990). Few studies have attempted to understand the mechanisms driving this behavior. In Chapter 2, I investigated physiological factors that could influence crop raiding in the African savanna elephant. I approached this question by collecting stress hormone and parasite load data on both crop raiding and non-crop raiding individuals and using those data to build a model that assesses the relationship between these variables and crop raiding behavior. This study took a unique perspective from that of the individual elephant, and will help to address a form of human-elephant conflict that frequently results in fatalities in humans and elephants alike.

In Chapter 3 I conducted a comparative analysis of the fecal microbiota of the African forest and savanna elephant. The vertebrate gut contains trillions of prokaryotic symbionts that aid in metabolic function (Savage 1977, Stevens and Hume 1998). Research has shown that the gut community composition is influenced by many variables including host phylogeny, diet and behavior (Ley, Hamady et al. 2008, Yamano, Koike et al. 2008). However, little work has been done to elucidate these relationships on non-model species outside controlled laboratory experiments. To make comparisons

between the microbiota of the two African elephant sister species, I sequenced a fragment of the 16S ribosomal RNA gene and assigned taxonomic identification to the sequences obtained. In addition, I conducted beta diversity analyses to assess the variation of the microbial communities between the individuals sampled. One implication of this work is to determine if the results we find in model species are applicable in a larger context and help to explain the role and evolution of the microbiota in wildlife species.

Although African forest and savanna elephants are some of the most visible of the "charismatic megafauna", there have been surprisingly few studies of these species using genetic techniques. Collecting samples from wild populations is challenging, and the logistics of transporting tissues or dung samples for analysis in the laboratory can be daunting. However, genetic studies provide important insights into the ecology, evolution and behavior of these species that would be difficult to obtain using other methods. In the face of habitat loss, fragmentation, and the continuous threat of illegal killing for ivory, effective management will become increasingly important. The data I obtained will aid in the development of strategies to manage populations of these keystone species.

CHAPTER 2:
EVIDENCE OF POSITIVE SELECTION IN MITOCHONDRIAL COMPLEXES I AND V OF THE AFRICAN ELEPHANT

Abstract

As species evolve, they become adapted to their local environments. Detecting the genetic signature of selection and connecting that to the phenotype of the organism, however, is challenging. Here we report using an integrative approach that combines DNA sequencing with structural biology analyses to assess the effect of selection on residues in the mitochondrial DNA of the two species of African elephants. We detected evidence of positive selection acting on residues in complexes I and V, and used homology protein structure modeling to assess the effect of the biochemical properties of the selected residues on the enzyme structure. Given the role these enzymes play in oxidative phosphorylation, we conclude the selected residues may affect the overall energy production and metabolism of these species. These amino acid changes may have contributed to the adaptation of forest and savanna elephants to their unique habitats.

Introduction

One of the central questions in molecular evolution revolves around whether natural selection at the DNA sequence level can be linked to adaptive phenotypic changes in the organism (Smith and Eyre-Walker 2002). Genetic mutations in protein coding genes can affect the folding and 3-D structure of the protein produced, creating a

cascade that may alter protein-protein interactions and modify biochemical pathways and cellular processes, all of which could affect the phenotype of the organism in a way that would impact its fitness (Dalziel, Rogers et al. 2009). Given the unique selective pressures of the environment in which an organism lives, those changes that confer fitness benefits may become fixed adaptations within a species over time. To elucidate the relationship between genetic variation and adaptive phenotypic traits, we adopted an integrative approach that combined detection of a molecular signature of selection with structural biological analyses to assess how the genetic changes affect the resulting protein and downstream networks that can be linked to adaptive phenotypic traits (Figure 1).

The mitochondrial genome (mtGenome) is an excellent system in which to study adaptive evolution. The 13 protein-coding genes in the mammalian mtGenome, along with dozens of nuclear genes, encode the protein subunits that make up four out of the five complexes of the electron transport chain (ETC) where the oxidative phosphorylation (OXPHOS) pathway occurs. OXPHOS plays a crucial role in energy metabolism and heat production, and through this pathway, mitochondria produce the majority of ATP that drives cellular processes. As a result, these proteins are under high functional constraint. However, given that metabolic requirements vary greatly across species, different selective pressures may be acting on these conserved complexes that lead to adaptive modifications.

The evolutionary history and phenotypic variation of the family Elephantidae make it an appropriate system for studying the adaptive evolution of the mtGenome in a long-lived, free-ranging mammal. The recent acquisition of whole mtGenomes for the extinct woolly mammoth (*Mammuthus primigenius*) and the American mastodon (*Mammut americanum*) have allowed for mitogenomic analyses of phylogenetic relationships among these taxa (Krause, Dear et al. 2006, Rohland, Malaspinas et al. 2007). The results of those studies suggest that the woolly mammoth and Asian elephant diverged shortly after diverging from their common ancestor with the African elephant. Mitogenomic and nuclear analyses of the taxonomy within *Loxodonta* suggest that the African savanna elephant (*Loxodonta africana*) and the African forest elephant (*Loxodonta cyclotis*) diverged approximately 5.5 million years ago (Roca, Georgiadis et al. 2001, Brandt, Ishida et al. 2012).

Ecological and morphological differences between African forest and savanna elephants result in differing metabolic requirements. African forest elephants are found in the tropical forest regions of West and Central Africa, and eat a diet largely of browse and fruits that includes a great diversity of plant species (White, Tutin et al. 1993, Lister 2013). In contrast, African savanna elephants are distributed in the savannas of eastern and southern Africa, and are generalist grazers/browsers that consume 60-95% of their forage as grasses (Owen-Smith 1988, Codron, Codron et al. 2011). Additionally, forest and savanna elephants are morphologically distinct, with forest elephants having a

substantially smaller body size than their savanna counterparts, shorter and rounder ears, and thinner, straighter tusks (Sikes 1971).

The selective neutrality assumption of mtDNA has been empirically tested and refuted across a broad range of organisms (Rand and Kann 1998). Recent studies have found evidence for molecular adaptations in the 13 protein-coding genes in the mtGenome (Ruiz-Pesini, Mishmar et al. 2004, Bazin, Glémin et al. 2006). Some mutations have been associated with pathogenic disorders in humans and mice including exercise intolerance, neurological diseases and myopathy (Wallace 1992, Rankinen, Bray et al. 2006), while others have been shown to have positive outcomes including greater aerobic energy metabolism (Grossman, Schmidt et al. 2001). In elephants and humans, Goodman et al. (Goodman, Sterner et al. 2009) show support for adaptively evolved mitochondrial functioning genes in the evolution of larger brain size and brain oxygen consumption. Considering the important role mitochondria play in metabolism, we might expect that some mutations in the mtDNA will result in ecological adaptations. When comparing the sequences of the protein-coding genes of the mtGenome across 41 mammal species, da Fonseca et al. (2008) found great variation in the biochemical properties of amino acids at functional sites, concluding that these changes may be adaptive to the special metabolic requirements across the diverse taxa. Research on anthropoid primates found an accelerated rate of non-synonymous substitutions in mtDNA that are linked to phenotypic changes, such as an enlarged neocortex and extended lifespan (Grossman, Wildman et al. 2004). Most recently,

research on Pacific salmon (genus *Onchorhynchus*) identified multiple sites within mitochondrial genes that were under positive selection and examined those sites in a structural context based on crystallized bacterial protein complexes (Garvin, Bielawski et al. 2011).

The five enzyme complexes of the ETC are embedded within the inner mitochondrial membrane. Four of these complexes contain varying numbers of mitochondrial encoded subunits in their structure. Complex I includes the seven subunits encoded by the NADH dehydrogenase (ND) genes (*ND1*, *2*, *3*, *4*, *4L*, *5*, *6*), the cytochrome *b* (*CYTB*) subunit is found in complex III, complex IV contains the three cytochrome oxidase (COX) gene subunits (*COXI*, *COXII*, *COXIII*), and lastly, the ATP synthase 6 (*ATP6*) and ATP synthase 8 (*ATP8*) subunits make up part of complex V. As electrons are passed through the series of five complexes, a proton-motive force is created to drive the synthesis of ATP from ADP and inorganic phosphate (Abrahams, Leslie et al. 1994).

Knowing the native state of a protein allows for a more powerful analysis of the biochemical properties that may affect the structure and, ultimately, the function of that molecule. Homology protein structure modeling is a useful tool that involves taking the known 3-D structure of a closely related protein and using it as a template to model an unknown protein structure (Sánchez and Šali 2000). Because changes in the protein sequence can produce changes in the 3-D shape, the objective of this study was to

investigate adaptive changes within African elephants by identifying regions of the mitochondrial genome that may be under positive selection and to use homology protein structure modeling to assess whether these changes may alter the structure or function of the protein. This is the first study to take an integrated approach using selection analyses and structural biology to predict 3-D structures of the OXPHOS proteins for the African elephant to identify adaptive sites in the mtGenome (Figure 1). Furthermore, we are the first to look for evidence of positive selection between the African forest and savanna elephant. Previous work has focused solely on the savanna elephant, but we utilize the most complete dataset of available forest elephant mtGenome sequences, including two individuals sequenced from dung samples. As such, we provide a framework by which studies on adaptive evolution can be undertaken on free-ranging wildlife species that may be more easily studied through noninvasive sampling techniques.

Results

Sequence and Phylogenetic Analyses

We sequenced 16,536 bp of the mitochondrial genome from a West African forest elephant and 16,541 bp from a Central African forest elephant. Start and stop codons in the forest elephant samples for each of the 13 protein coding genes are shared with those of the reference savanna elephant mtGenome (AB443879.1) (Murata, Yonezawa et al. 2009). The only sequence anomaly, also noted by Brandt et al. (2012), is a 2 bp insertion in the *12S rRNA* gene for the Central African forest elephant that is not

found in other elephantid mtGenomes. Relationships within the Elephantidae using the complete mtGenome are depicted in Figure 2. Excluding the clade of mammoths, the posterior probability for each clade is 1. In addition to the monophyly of *Loxodonta*, our findings confirm the deep divergence between African forest and savanna elephants (Brandt, Ishida et al. 2012). This is the first study to sequence the entire forest elephant mtGenome from dung samples. This serves as a proof of concept for future research in this area that aims to focus on noninvasive sampling of free-ranging wildlife species that may be of conservation concern.

Adaptive Evolution Analysis

Analysis in TreeSAAP identified several significant amino acid changes. Those that differ between forest and savanna elephants are found in complexes I and V of the ETC. In complex I, we found six significant changes between forest and savanna elephants in the *ND1*, *ND4*, *ND5* and *ND6* genes, and two in the *ATP6* gene of complex V (Table 2). As a result, we focused further analyses on these two complexes.

Both complexes I and V contain domains in the inner mitochondrial membrane. It is very challenging to solve tertiary structures of transmembrane proteins, but two homologous bacterial structures were found in the Protein Data Bank (PDB) (Berman, Westbrook et al. 2000) for complex I: one for *Thermus thermophilus* (PDB ID: 4HE8) (Baradaran, Berrisford et al. 2013) and the other for *Escherichia coli* (PDB ID: 3RKO) (Efremov and Sazanov 2011). No high resolution homologous structures were found for

complex V. Therefore, we proceeded with homology modeling analyses for complex I, but not for complex V.

Complex I Structure and Function

Complex I is the first and largest enzyme complex in the OXPHOS pathway, and mutations in its subunits have been linked to many human neurodegenerative diseases (Wallace 1992). This complex is known to be one of the largest membrane protein assemblies with 44 subunits comprising the eukaryotic complex, 14 of which are homologous to bacterial subunits and provide a catalytic core of the enzyme (Carroll, Fearnley et al. 2006, Balsa, Marco et al. 2012). It catalyzes the reactions that synthesize ATP by creating an electrochemical proton gradient. First, NADH is oxidized in the mitochondrial matrix, which provides two electrons to be transferred to quinone in the inner mitochondrial membrane (Walker 1992). This electron transfer is coupled with pumping four protons across the inner mitochondrial membrane, thus producing an electrochemical gradient. While no crystal structure of complex I from a multicellular eukaryote has been obtained, images from low-resolution electron microscopy have revealed that the eukaryotic complex I forms an L-shaped structure with a membrane arm embedded within the inner mitochondrial membrane and a peripheral hydrophilic arm that protrudes into the mitochondrial matrix (Radermacher, Ruiz et al. 2006).

Complex I is encoded by both nuclear and mitochondrial genes. The membrane domain in *T. thermophilus* confirms that the homologous eukaryotic subunits encoded

by mtDNA genes *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*, are found in the membrane arm (Baradaran, Berrisford et al. 2013). Similar results have been shown for the *E. coli* complex I structure, although the homologous subunit encoded by *ND1* was not crystallized because it readily dissociates from the complex (Efremov and Sazanov 2011). It is believed that the coupling mechanism, by which the electrochemical gradient is created, occurs due to long-range conformational changes. Baradaran et al. (2013) propose that the quinone-binding site is found at the interface of subunit *ND1* and the hydrophilic arm. Subunits *ND1*, *ND6* and *ND4L* form a proton-translocation channel that ejects a proton into the periplasm. During each cycle, three additional protons are transferred into the periplasm by proton pumps encoded by subunits *ND2*, *ND4* and *ND5*. Subunit *ND3* is thought to intertwine with *ND1* in order to stabilize the interface between the membrane and hydrophilic domains.

African Elephant Complex I Structure

After homology modeling and side chain refinement, free loops that were not aligned with either of the two template structures were omitted, resulting in the final tertiary structure model for the African elephant complex I shown in Figure 3a. Root-mean-square deviation (RMSD) values and a TM-score were calculated as a quality assessment of the structure. As a comparison, a RMSD of 3.39 Å was found for 1,814 amino acid residues on the aligned chains (N, A, M, K, L, J) of the *T. thermophilus* and *E. coli* templates, and the TM-score between these two structures was 0.881. The 1,546 residue alignment of the savanna elephant structure with that for *T. thermophilus*

resulted in a RMSD of 7.31 Å and a TM-score of 0.596, while the RMSD for the 1,371 residue alignment with *E. coli's* structure produced a value of 6.61 Å and a TM-score of 0.592. Considering the large size of the structure and the RMSD value between the two bacterial templates, the RMSD values for the elephant model demonstrate support for our predicted structure, as do our TM-scores, which are all greater than 0.5.

For the four forest elephant samples included in this study, there are three possible combinations of mutations that are mapped onto our African elephant complex I structure (Figure 3b). Figure 4 shows the atomic structure for each of those mutations. To estimate whether the selected residue was buried inside the protein or on the surface, we calculated relative accessible surface areas (ASA) for each of the mutations. We applied a 5% threshold on accessibility to define whether a residue was found on the surface or was buried (Miller, Janin et al. 1987). As such, we found that three of the mutation locations (*ND1,49, ND5,20*, and *ND6,45*) had values higher than 5% and are on the surface of the protein (Table 3). Four of the mutation locations have at least one chain-chain binding site for the mtDNA encoded subunits. It is possible these residues could interact with the nuclear encoded subunits that have not been sequenced.

Structural and Functional Effects of Selected Residues in Complex I

The alignment of homologous structures for complex I reveals that each of the six significant mutations found in this study are in regions that are not highly conserved across species (Efremov, Baradaran et al. 2010, Efremov and Sazanov 2011, Baradaran,

Berrisford et al. 2013). Based on the alignment of our *L. africana* complex I structure with that of *T. thermophilus*, we determined the location of our selected residues within the protein chains. Chain *ND1* has 9 transmembrane (TM) helices. The mutation at *ND1, 49* is located in TM1, which creates part of a narrow entryway for the quinone. Here, the forest elephant sample from CF has a valine while all other forest and savanna samples share an isoleucine. According to the Taylor classification (Taylor 1986), both of these amino acids are aliphatic, hydrophobic residues, so we would not expect this substitution to result in large structural changes. However, given its location near the quinone-binding site and because it is predicted to be both a surface residue and interact with subunits *ND2* and *ND3*, this may affect the overall conformation and/or efficiency of the entry point for the quinone molecule. Near subunit *ND1* and forming part of the fourth proton-translocation channel are two significant substitutions located at binding sites on subunit *ND6*, which contains five TM helices. At *ND6, 43*, located in TM2, savanna elephants along with the forest elephant SL sample display isoleucine whereas the other three forest elephants sampled have a valine. As described above, isoleucine and valine share similar biochemical properties. This site interacts with residues on three other chains encoded by *ND2*, *ND3*, *ND4L*, thus making it more likely to impact the overall structure of the proton-translocation channel *ND6* forms with subunits *ND1* and *ND4L*. Savanna elephants share a glycine at *ND6, 45*, which is found in the loop region between TM2 and TM3, while all forest elephant samples have a serine. Both of these amino acids are small, but serine is a polar residue and glycine is hydrophobic. This buried residue is at a protein binding site for chains *ND2* and *ND4L*,

which may cause conformational changes for the proton-translocation channel and affect its efficiency. The remaining three substitutions are part of the membrane-bound proton pumps. Of the 14 TM helices in subunit *ND4*, position 15 is located in TM1 where it was found that savanna elephants have alanine while forest elephants from CI, GA and SL share a threonine residue and the sample from CF has valine. All three of these residues are small, but alanine is non-polar and slightly hydrophobic, valine is aliphatic and more hydrophobic, and threonine can be both polar and hydrophobic. This substitution is found on a binding site for another of the proton pumps encoded by gene *ND2* and forms part of a lipid-facing layer. Subunit *ND5* has significant substitutions at positions 20 and 21, both of which are found in TM1 (there are 16 total) that is also part of the lipid-facing layer. At residue 20, savanna elephant samples share an isoleucine and all forest elephant samples have threonine. Both residues can be hydrophobic with isoleucine classified as aliphatic and threonine also being polar. Lastly, at site 21, all savanna elephants and forest samples from CI and GA have threonine, while the SL forest elephant sample has alanine and CF has isoleucine. As previously described, isoleucine is the most hydrophobic residue and is also aliphatic, while alanine is less hydrophobic and polar, and threonine is polar. Although the amino acid substitutions observed between forest and savanna elephants at the proton pumps are not that unlike in their biochemistries, they are at locations that could alter the efficiency of the pumps, thus affecting the OXPHOS pathway and resulting in phenotypic changes between species. Mutations that affect a protein's interaction with other proteins that form a biochemical pathway are capable of altering the phenotype (Dalziel, Rogers et al.

2009). Four out of six of our selected mutations (Table 3) are at protein-binding sites and are likely to have a great effect on the OXPHOS pathway and adaptive evolution of forest and savanna elephant species.

Complex V Analyses

Complex V, or ATP synthase, was the other enzyme in OXPHOS where we identified significant amino acid changes between the forest and savanna elephant. The role of ATP synthase in OXPHOS is to phosphorylate ADP to synthesize an ATP molecule. ATP synthase is composed of two distinct units: the water soluble F1 portion that contains the catalytic sites and the transmembrane F0 portion that acts as a proton turbine (Arsenieva, Symersky et al. 2010).

We found two significant sites in the *ATP6* gene, which codes for subunit *a*, that is thought to participate directly in the proton flow (Arsenieva, Symersky et al. 2010). Because of the difficulty in crystallizing membrane proteins, little information is known about the structure of the F0 proton channel (Weber and Senior 1997) and therefore we have not conducted further structural analyses. We can, however, look at the biochemical differences for the residues of interest. At site seven of the *ATP6* gene *L. africana* has a threonine while all *L. cyclotis* samples have an alanine. Both are small residues, but threonine is polar and alanine is non-polar. Perhaps the greatest biochemical difference between amino acid substitutions is found on *ATP6* site 10 where savanna elephants share a tyrosine and the forest elephants have aspartic acid.

Tyrosine has an aromatic side chain, is slightly hydrophobic and polar, while aspartic acid is also polar, but has a negative charge. In this complex, large conformational changes are required to occur in order to couple the passage of protons with the production of ATP. As a result, the selected amino acid substitutions between forest and savanna elephants could affect these conformational changes and alter the efficiency of ATP production, and thus metabolism, in these two species.

Discussion

The 13 protein-coding genes of the mtGenome code for the machinery that make up the complexes of the ETC, which is a key biochemical pathway involved in the production of ATP and consequently is closely linked to metabolic activity. The objective of this study was to compare mtGenome sequences between the African forest and savanna elephant in order to identify sites in the mtGenome that might be under positive selection and result in adaptive differences between these two species. To accomplish this, we used an integrative approach that combined sequencing and structural genomic techniques to provide insights on how the selected residues might affect protein structure and function of the OXPHOS pathway. We argue that these changes relate to metabolic adaptations in *Loxodonta*.

Our results are in line with other studies that have found evidence of adaptive evolution in the ETC complexes (Xu, Luosang et al. 2007, da Fonseca, Johnson et al. 2008, Garvin, Bielawski et al. 2011). Garvin et al. (Garvin, Bielawski et al. 2011) detected

a strong signal of positive selection in the *ND2* and *ND5* genes between species of Pacific salmon. Specifically, they linked the significant sites on the *ND5* gene to the structural piston arm of a proton pump and suggest the possibility that changes in the proton pump may have influenced fitness during the evolution of the salmon species studied. In an analysis of 41 mammalian species, da Fonseca et al. (da Fonseca, Johnson et al. 2008) found evidence of positive selection in the three proton pumps encoded by genes *ND2*, *ND4* and *ND5*. Research studies on equids argue that mutation patterns in the *ND6* gene are indicative of an adaptation to high altitude (Xu, Luosang et al. 2007, Ning, Xiao et al. 2010).

While residues are conserved amongst *L. africana*, we see variability in the residues found in *L. cyclotis*. This finding might be expected given the higher genetic diversity known to occur in forest elephants (Roca, Georgiadis et al. 2001). Phylogeographic studies of forest elephants using mitochondrial DNA suggest that their evolutionary history is more complex than just grouping them into a separate species from their savanna counterparts (Eggert, Rasner et al. 2002, Johnson, Clifford et al. 2007). A similar study on killer whales (*Orcinus orca*) found evidence of positive selection in the *CYTB* gene between two distinct ecotypes, and suggests these amino acid substitutions are ecological adaptations. In addition, empirical research on sympatric haplotypes of *Drosophila simulans* suggest that mtDNA variation is responsible for phenotypic differences that include cold tolerance, starvation resistance and greater egg size and fecundity (Ballard, Melvin et al. 2007). The varying selective

pressures acting on populations of the same species under differing environmental conditions may lead to specialized metabolic adaptations in the mitochondrial genes that code for the OXPHOS pathway that functions to synthesize ATP and generate heat to maintain body temperature.

The morphological and ecological differences between the forest and savanna elephant could influence their respective metabolic requirements. Standard metabolic rate is a good descriptor for the minimal rate of energy flow for an animal. Based on the empirically tested equations for standard metabolic rate, it has been shown that, in general, larger organisms respire at a higher rate than smaller organisms (Peters 1986). Forest elephants have a more compact body stature than their savanna counterparts with one population comparison finding *L. cyclotis* to be 35-40% shorter than *L. africana* (Morgan and Lee 2003), thus, they consume less oxygen. One study on leukaemic cells linked mutations in the *ND1* gene to increased levels of oxygen consumption (Piccoli, Ripoli et al. 2008). This combined with other evidence offering support for adaptive evolution in OXPHOS proteins related to higher brain oxygen consumption in elephants (Goodman, Sterner et al. 2009) suggests that our results on the selected amino acid substitutions between *Loxodonta* species could equate to phenotypic differences in oxygen production and consumption.

In addition, thermoregulation plays an important role in the biology and adaptation of the African elephant. As with standard metabolic rate, metabolic heat

production scales with biomass where larger mammals have lower body temperatures (McNab 1983). Larger animals also have smaller surface area: volume ratios, resulting in less area available for heat transfer (Williams 1990). This physiological constraint is compacted even further for the savanna elephant given it inhabits hot, arid environments where seasonality causes extreme fluctuations in water and food availability. The forest elephant, however, experiences less dramatic inter-seasonal variation in its tropical closed-canopy forest habitat. Given the role of the OXPHOS pathway to generate heat and maintain body temperature, residue substitutions that reduce the coupling efficiency of ATP synthase would result in lower ATP production and increase heat production (Mishmar, Ruiz-Pesini et al. 2003). The two mutations we found in complex V between forest and savanna elephants may affect the coupling efficiency and could be adaptation to their different thermoregulation requirements.

This research provides a framework for studying adaptation of the OXPHOS pathway in non-model species. When working with free-ranging wildlife of conservation concern, it is often impractical and unethical to conduct empirical studies. We show that it is possible to collect noninvasive field sample to carry out meaningful selection and structural biology analyses. While we are limited in our capacity to test for the impact certain mutations have on physiology and function, we believe the changes we found in the mitochondrial genome for forest and savanna elephants play a role in their adaptive evolution.

We have taken a novel approach to studying the adaptive evolution of the mtGenome by combining phylogenetic and protein prediction methods to better understand the structural and functional biology of the OXPHOS pathway in the African elephant. This is the first study to predict the protein structure from any of the ETC complexes for a specific study species to more accurately identify the locations of our selected residues. Given the lack of a high resolution structure for complex V, we were unable to use computational biology tools to predict the homologous structure for the African elephant. Nonetheless, our results provide evidence for the adaptive evolution of African forest and savanna elephants to their specialized morphologies and ecologies. Future work includes sequencing the nuclear genes that code for protein subunits that complete the machinery for the OXPHOS enzyme complexes to better understand the protein interactions and how they might lead to functional changes between the species. Additionally, we aim to sequence samples spanning the range of *Loxodonta* to identify associations between adaptive changes and landscape features, such as in Foote et al.'s work (2010), as well as phylogeographic patterns.

Materials and Methods

Samples

Dung samples from African forest elephants were originally collected at Taï National Park, Cote d'Ivoire (CI), and Lopé National Park, Gabon (GA) as part of population level studies (Eggert, Rasner et al. 2002, Schuttler 2012). We selected one sample from each park to sequence, therefore giving us two novel forest elephant

mtGenome sequences for our study. These locations are deep within the forest zones of West and Central Africa, thus avoiding regions in which historical or contemporary hybridization may have occurred between forest and savanna elephants (Roca and O'Brien 2005). Approximately 20 g of dung were collected and boiled in the collection tube to prevent the transportation of pathogens, then stored in Queens College preservation buffer (20% DMSO, 0.25 M EDTA, 100 mM Tris, pH 7.5, saturated with NaCl(Amos, Whitehead et al. 1992)). Total genomic DNA was extracted from dung samples in a lab dedicated to noninvasive DNA extractions (Eggert, Maldonado et al. 2005) using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen, Valenica, CA, USA) with modifications as described in Archie (Archie, Moss et al. 2006). In addition, we used previously published whole mtGenome sequences for members of the Elephantidae (Table 1).

DNA Amplification and Sequencing

We designed 44 primer pairs using a savanna elephant sequence (Acc # AB443979.1) as a template. Fragment sizes varied between 175 and 522 bp, and covered the entire mitochondrial genome excluding a variable number of tandem repeats (VNTR) found in the control region (Table 4). To sequence both ends of the VNTR, we amplified and cloned a 136bp fragment using a Topo TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Ten clones per forest elephant sample were purified using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced at the University of Missouri's DNA CORE in a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

For all other fragments PCR was performed using an Eppendorf Mastercycler ep thermocycler in 25 µL volumes containing 1 X PCR gold buffer, 0.2 µM dNTP, 0.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1.5 mM $MgCl_2$, 10 X BSA (New England Bioloabs, Ipswich, MA, USA), 0.4 µM forward primer, 0.4 µM reverse primer, and 2 µL of DNA template. The profile included an initial denaturation step at 95°C for 10 minutes, followed by 50 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 1 minute, and primer extension at 72°C for 1 minute, ending with an elongation step at 72°C for 10 minutes. A negative control sample was included with every PCR to detect contamination of reagents. Amplification products were visualized in a 2% agarose gel and fragments of the correct length were purified with a QiaQuick PCR purification kit (Qiagen) and sequenced on a 3730xl 96-capillary DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the University of Missouri's DNA CORE facility.

Phylogenetic Analyses

Sequences were assembled and aligned using Sequencher v. 4.5 (GeneCodes, Ann Arbor, MI). As nuclear insertions of mtDNA (numts)(Bensasson, Zhang et al. 2001) are commonly found in elephant DNA extracted from hair samples (Greenwood and Pääbo 1999), we examined the translation of all protein coding sequences to verify the open reading frame. We aligned both forest elephant sequences to 5 mammoth, 3 Asian elephant and 3 savanna elephant sequences available in GenBank (Benson, Cavanaugh et al. 2013), thus bringing the dataset to 15 individuals (Table 1). The mammoth was selected as an outgroup for phylogenetic analyses.

After inferring phylogenetic relationships using each of the 13 protein coding genes (*ATP6*, *ATP8*, *COX1*, *COX2*, *COX3*, *CYTB*, *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, *ND6*), we ran a concatenated data set with 15 partitions: each of the 13 protein coding genes, all tRNAs, and both rRNAs. Since using a single model of evolution for the entire mtDNA sequence may result in error, we selected a model of evolution for each partition using FindModel (Table 5) (Tao, Bruno et al. 2005). When certain samples (typically mammoth) had more amino acids than other taxa, protein coding gene alignments were edited to be the same length. To infer phylogenetic relationships among the 15 sequences, Bayesian inference with Markov chain Monte Carlo (MCMC) sampling was conducted using MrBayes v. 3.1 (Huelsenbeck, Ronquist et al. 2001, Ronquist and Huelsenbeck 2003). The combined total alignment for the partitioned dataset was 15,354 bases including a 2 bp insertion in the 12S rRNA gene for the forest elephant samples from Gabon and the Central African Republic (CF). We ran 3 chains for 10,000,000 generations with trees being sampled every 1,000 generations. To infer phylogenetic relationships using maximum likelihood we used PhyML 3.0 (Guindon, Dufayard et al. 2010).

Adaptive Evolution Analyses

A common method to detect selection in protein coding genes is to estimate ω, the non-synonymous to synonymous rate ratio model (Yang 1998), but this method is highly conservative and biased against detecting positive selection when a select few

amino acid changes may result in adaptive changes. Due to the conserved nature of the mitochondrial genome, we used the algorithm implemented in TreeSAAP (Selection on Amino Acid Properties) (Woolley, Johnson et al. 2003) to identify significant amino acid changes among the members of Elephantidae. TreeSAAP compares the distribution of observed changes inferred from a phylogenetic tree with the expected random distribution of changes under neutral conditions. To test for significant amino acid changes in our dataset, we analyzed the phylogenetic tree for each of the 13 protein coding genes separately. TreeSAAP utilizes a sliding window to analyze the magnitude of change for 31 physicochemical properties of amino acids and rates those substitutions on a scale of 1 (most conservative) to 8 (most radical). A significant positive z-score for any of the physicochemical properties included in the analysis indicates more non-synonymous substitutions than are expected under neutral conditions, suggesting positive selection. We included all 31 physicochemical properties, set our sliding window equal to 15 codons, and considered only the most radical amino acid substitutions (categories 7-8, $p \leq 0.001$).

Protein Structure Prediction and Analysis

Complex I is a large assembly consisting of seven mtDNA-encoded subunits, which are covered by one or two structural templates. Due to relatively low sequence-identities (18-42%, Table 6) between the sequences of the constituting protein subunits and their structural templates, we used a hybrid comparative approach to model the structure of the overall complex.

First, the protein sequences of the individual subunits for *L. africana* were aligned with the corresponding sequences of homologous subunits from both template structures, *T. thermophilus* and *E.coli*. MODELLER (Sali and Blundell 1994) was used to predict the tertiary structure for the mtDNA-encoded individual subunits (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5*, *ND6*) of complex I in the African elephant. Second, we used Chimera (Pettersen, Goddard et al. 2004) molecular structure visuallization software to generate the overall structure of the savanna elephant complex I by structurally aligning individual subunits against complex I templates from *T. thermophilus*. Third, FoldX (Schymkowitz, Borg et al. 2005) structure refinement software was used to refine the modeled complex I by adjusting side chains to result in lower free energy levels, thus creating a more stable structure. Finally, to assess the quality of the modeled complex structure, we structurally aligned the model of complex I with each template structure to measure the RMSD value and the TM-score in TM-align (Zhang and Skolnick 2005). The RMSD value represents the average deviation between the corresponding residues of two proteins. Smaller values indicate higher similarity between structures, and values increase as the length of the protein chain increases. Similarly, the TM-score assesses the topological similarity between two protein structures and produces an output between [0,1] with higher values indicating better models (Zhang and Skolnick 2004).

Once we modeled complex I for the African elephant, we calculated relative ASA values for each residue identified to be under positive selection using NACCESS

(Hubbard and Thornton 1993) and determined whether the residues were located at chain-chain binding sites with FoldX (Table 3). ASA values represent the area of the residue that is in contact with the solvent and is used to distinguish the protein surface from the interior (Miller, Janin et al. 1987).

Table 1: Accession numbers for samples included in this study

Species	Sample Name	Accession #	Citation
Mammuthus primigenius			
	Mammoth 1	AP008987	Ozawa et al. unpublished
	Mammoth 2	DQ316067	Rogaev et al. 2006
	Mammoth 3	EU155210	Gilbert et al. 2008
	Mammoth 4	NC_007596	Krause et al. 2006
	Mammoth 5	DQ188829	Krause et al. 2006
Elephas maximus			
	Asian 1	DQ316068	Rogaev et al. 2006
	Asian 2	NC_005129	Rogaev et al. 2006
	Asian 3	EF588275	Maikaew et al. unpublished
Loxodonta africana			
	Savanna 1	AB443879.1	Murata et al. 2009
	Savanna 2	NC_000934.1	Hauf et al. 2000
	Savanna 3	DQ316069.1	Rogaev et al. 2006
Loxodonta cyclotis			
	CI	This study	This study
	GA	This study	This study
	SL	JN673264	Brandt et al. 2012
	CF	JN673263	Brandt et al. 2012

Table 2: Significant amino acid changes in the mitogenome identified by TreeSAAP

Complex	**Gene/Position**	***Mammuthus primigenius***	***Elephas maximus***	***Loxodonta africana***	***Loxodonta cyclotis:*** **CI**	**GA**	**CF**	**SL**
I	ND1, 49	I	I	I	I	I	V	I
I	ND4, 15	A	A	A	T	T	V	T
I	ND5, 20	T	T	I	T	T	T	T
I	ND5, 21	T	L	T	I	T	I	A
I	ND6, 43	I	I	I	V	V	V	I
I	ND6, 45	S	S	G	S	S	S	S
V	ATP6, 7	A	A	T	A	A	A	A
V	ATP6, 10	D	D	Y	D	D	D	D

CF = Central African Republic; CI = Cote d'Ivoire; GA = Gabon; SL = Sierra Leone.

Table 3: Interaction of residues with other protein subunits of Complex I as determined by the predicted African elephant protein structure.

Mutation	Binding Residue	Interacting Subunit(s)	Relative Accessible Surface Area (%)
ND1, 49	+	ND2, ND3	30.1
ND4, 15	+	ND2	0.2
ND5, 20	-	-	42.2
ND5, 21	-	-	0.8
ND6, 43	+	ND2, ND3, ND4L	0.1
ND6, 45	+	ND2, ND4L	54.0

Table 4: List of primer sequences used in this study, and the region they amplified in the forest elephant mitochondrial genome

Primer Name	Forward Sequence	Primer Name	Reverse Sequence	Bases covered (incl. primers)
MT1F	CACCATGCATATCACCTCCA	MT1R	GCCATAGCTGAATCACAGCA	15732-16023
MT2F	ATTTTGGGGATGCTGTGATT	MT2R	TGTGTGTACGCTGGGAATTT	15993-16166
MT3F	AAATTCCCAGCGTACACACATA	MT3R	GGGGTTTGAAGAGATAGTTACA	16149-16694 (incl. VNTR)
MT4F	CAAACCCCAAAAGCAGGACTAT	MT4R	GCTTGATGCCAGCTCTCTTT	16744-16950, 0-187
MT5F	AAAGAGAGCTGGCATCAAGC	MT5R	TCTCTGGCGGATAGCTTTGT	168-567
MT6F	ACAAAGCTATCCGCCAGAGA	MT6R	GCTTCATGGCCTTCAAT	571-905
MT7F	GTGGTTGAAGGCGGATTTAG	MT7R	CATCATTCCCTTGCGGTACT	841-1233
MT8F	TCAAAACATTCACTTACCAAAAAG	MT8R	AAAGAGCTGTACCCCTTTTGAA	1128-1578
MT9F	CCACAAAAACAAAATAATTCCAA	MT9R	TGCCTGTGTTGGGTTAACAA	1490-1871
MT10F	GGATGCCCGCTGATAGTTA	MT10R	GAGGTCACCCCAACCAAAAT	1812-2260
MT11F	CGAGAAGACCCTATGGAGCTTA	MT11R	TTTAGCTGGAGGCGTCTTAGT	2152-2611
MT12F	TCCAGTACGAAAGGACAGAAAA	MT12R	GAGGATATGGTATTGGAAGAGGA	2553-3039
MT13F	GCCCCAATTCTAGCCCTAAC	MT13R	TGATGATGTTAGCCCTAAC	2942-3449
MT14F	GAATACTCAGCTGGCCCTTTC	MT14R	CGTGGTGTAATTGGTAGCACTG	3382-3830
MT15F	CGAACCTAAACTCGAGAATTCAA	MT15R	TGGAACTCAGAAGTGGAATGG	3780-4255
MT16F	AACCAACCCTGTAGCATCAAA	MT16R	TGGTTATTGCAGGTGTTTTGT	4174-4631
MT17F	TTCACAACTTCAATATCATCCCTAA	MT17R	GCGTCTGATTTGCATTCAGTT	4574-5069
MT18F	CCTGAAATAAGGACTGCAAGA	MT18R	CCAAGAAGAGAGCCTGGTTG	5014-5452
MT19F	TTATTTGGTGCTTGAGCTGGT	MT19R	GCTGGAGGTTTTATGTTGATGA	5392-5856
MT20F	CCTTGCAGGAGTATCCTCTATTCT	MT20R	CGCCAGTTGGAATAGCAATAA	5773-6294
MT21F	GGCATAGACGTTGACACTCG	MT21R	TTCATGCAGTATAGGCATCTGG	6218-6683
MT22F	TTCCCCAACACTTTCTTGGA	MT22R	GGTGTGGTCATGGAAGTGAA	6600-7103
MT23F	ACAATTGGGGTTCCAAGATG	MT23R	ACAGCCCATGAATGGAGAAC	7037-7527
MT24F	CCTACAGACCTACCAGTTCGAG	MT24R	TCTTTCAAGGGAAAACACATGA	7455-7932

MT25F	TCGCCTTCTTTTCCCAATCT	MT25R	AGGTGTTCCTTGTGGGAGAA	7871-8336
MT26F	GTTGTCCTGGGTTTCCGATA	MT26R	AGCAGGAGGGTTACAGAGTGA	8250-8735
MT27F	CCCTACTTATAACATCCGGCCTA	MT27R	TCGGAGATTGTAAAGGATGC	8675-9173
MT28F	GGAGTCTACTTCACACTTCTCCAA	MT28R	AACGAGCAGAGCCTACTGGA	9112-9603
MTAF	GGGCCTCATTTCAGACTCAG	MTAR	GAGGCTATTTGACCAATAATGATG	14778-15097
MTBF	CACACACATTGGACGAAACA	MTBR	AATGGTATAGTACGGGTGAAAGG	14436-14829
MTCF	AAAACAATATACATCATTACTTCCACA	MTCR	TGCCGGTATTTCAAGTTTCC	14057-14500
MTDF	AGACGCTCCCCCAGAATAAT	MTDR	CAACGATGGTTTTTCAGATCA	13642-14127
MTEF	ATAGCCCTGGCTGTAACCTT	MTER	TCATGGTTCTTGGTGAAGAGG	13174-13723
MMFF	CCTCTGGCTCTATCATCCACA	MTFR	AAGTTCTGTTGCTACCGTAAATCC	12765-13252
MTGF	CCAACCCCAAGAACACTACA	MTGR	GTCCTCCTATTTTGCGGATG	12336-12824
MTHF	CACTGAATAACAATCCACACCCTA	MTHR	TGAGGCCAGGAGAAGACCTA	11939-12400
MTIF	CCTTTCCCTAAACCCCAAGA	MTIR	AGGGAAAAGAAGTCTAATTTAAAGC	11502-12016
MTJF	TTTCATGGGAGCACTTACCC	MTJR	TGTTTTGGTTAAACTATGTCTGCAA	11088-11615
MTKF	CCATACCTAATCAAACCCATTGA	MTKR	TTGAATTTGCTAGACAGAAAAGTATTG	10690-11179
MTLF	AGCCTAAACCTCTCCCCAGA	MTLR	GGTGGAGACCATATAGAGGTATTTTT	10298-10804
MTMF	GCCTTCGTTATTTCACTCATTF	MTMR	CAAATAGTAAGGATTAGTAGTGGGGTA	9929-10376
MTNF	TTCTAATCGCATTTTGACTACCC	MTNR	CGAAGATATTAGGTGAGAGCGGTA	9539-9961
MTYF	ACCACTATGTAACTATCTCTTCAAACC	MTYR	TGATTTGCTTTCACCCCTATG	16664-16838
MTZF	GCGTCCTAGCCCTACTCCTA	MTZR	GGTAGTTTTCGATTACTCCTGCAA	15020-15277
MDL 3	Fernando et al.[1]	MDL 5	Fernando et al.[1]	15164-15803

Table 5: The model of evolution used for each partition for phylogenetic analysis as determined by FindModel

Partition	Model of Evolution
ATP6	HKY
ATP8	HKY
COX I	GTR + G
COX II	HKY
COX III	GTR + G
CYT B	HKY + G
ND1	GTR + G
ND2	GTR + G
ND3	HKY + G
ND4	HKY + G
ND4L	HKY + G
ND5	GTR + G
ND6	GTR + G
tRNAs	GTR + G
rRNAs	GTR + G

Table 6: Values representing percent sequence identity and coverage between the African elephant and two structural templates for each of the mtDNA-encoded subunits modeled in Complex I.

Subunit	***E. coli* template**	**Sequence Identity (%)**	**Coverage (%)**	***T. thermophilus* template**	**Sequence Identity (%)**	**Coverage (%)**
ND1	-	-	-	4HE8 chain H	41	93
ND2	3RKO chain N	23	97	4HE8 chain N	29	61
ND3	3RKO chain A	34	93	4HE8 chain A	37	69
ND4	3RKO chain M	31	83	4HE8 chain M	31	52
ND4L	3RKO chain K	27	99	4HE8 chain K	29	99
ND5	3RKO chain L	39	70	4HE8 chain L	42	63
ND6	3RKO chain J	19	97	4HE8 chain J	18	96

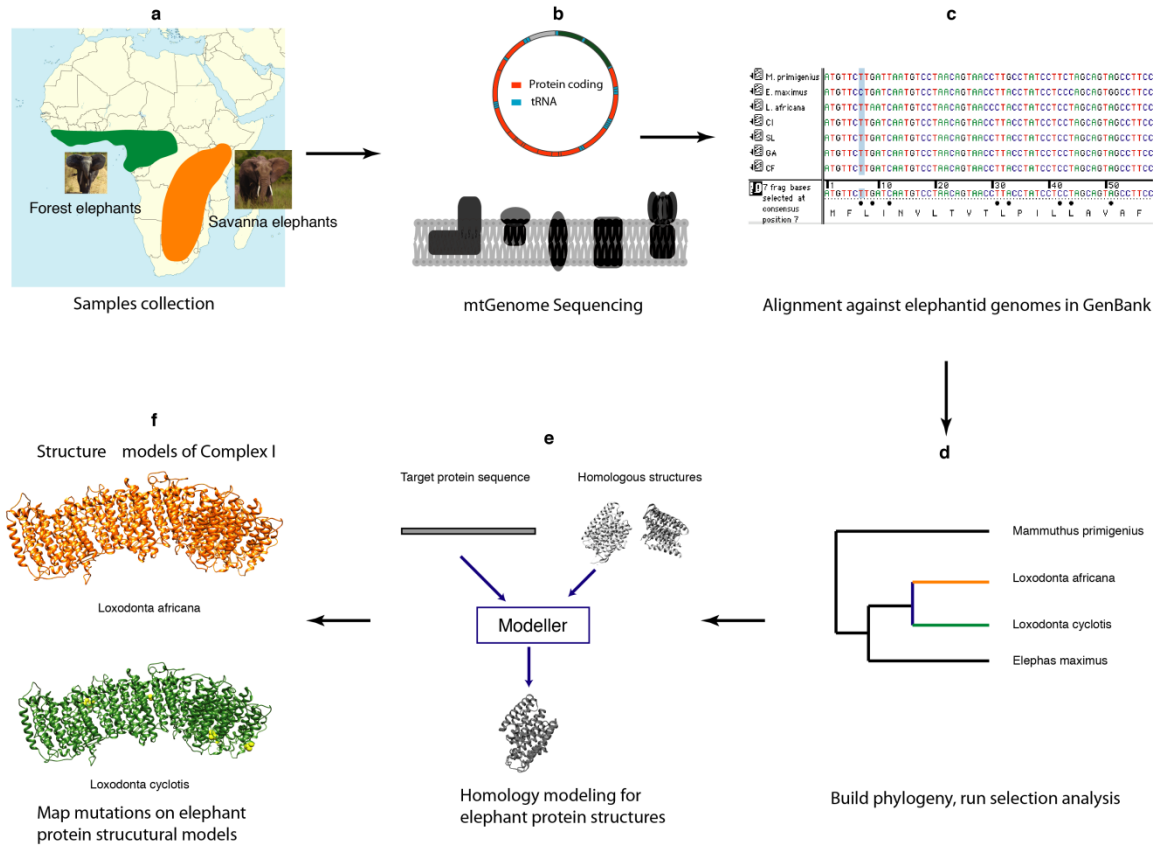


Figure 1: Flowchart outlining the methodological steps taken in our integrative approach to identify and analyze the structural biology of sites in the mitochondrial genome under positive selection in the African elephant. (a) Sample collection; green shows the range of the forest elephant (L. cyclotis) and orange shows the range of the savanna elephant (L. africana). (b) Sequencing the mtGenome; the protein coding genes encode for the subunits of the complexes involved in OXPHOS as shown in cartoon form. (c) Sequence alignment; complete mtGenome sequences for members of the Elephantidae were downloaded from GenBank, and to which we aligned our novel forest elephant sequences. (d) Phylogenetic and selection analyses; we inferred a phylogeny from our complete, aligned mtGenome sequence data and used the output to run analyses identifying sites that might be under positive selection. (e) Homology protein modeling; after identifying which genes (and complexes) might have sites under position selection, we searched the Protein Data Bank for homologous crystal structures, then input our elephant sequences and used Modeller to predict the elephant protein structures. (f) Mutation mapping; lastly, we mapped the residues that might be under positive selection onto our predicted elephant protein structures and assessed what impacts those substitutions found between L. cyclotis (green) and L. africana (orange) might have on the function of the protein in order to relate that to biological differences.

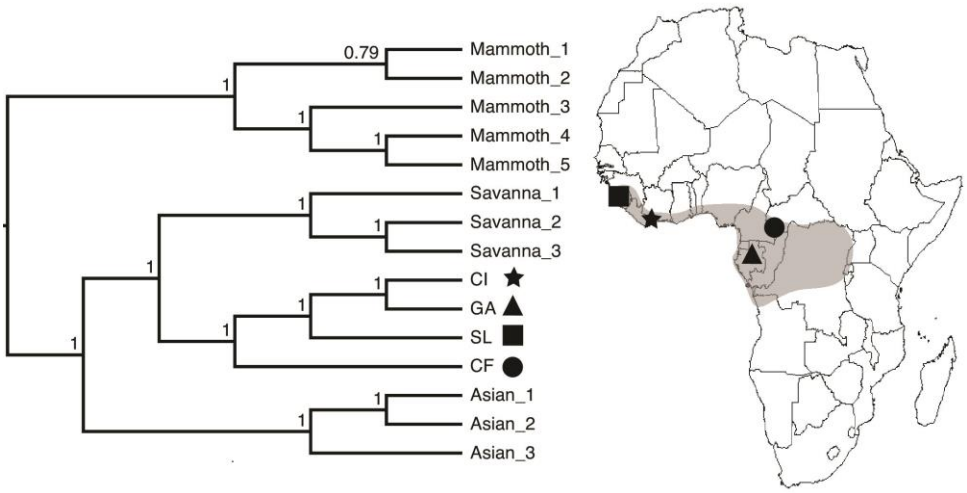


Figure 2: Whole mtGenome phylogeny for samples included in this study. Results from MrBayes are presented (PhyML shows same topology; 15,400 bp, 15 partitions) alongside a map of Africa showing the origin for the forest elephant samples (shaded area represents present-day forest zone). The star represents Taï National Park, Cote d'Ivoire (CI); triangle represents Lopé National Park, Gabon (GA); square represents Sierra Leone (SL); and circle represents Dzanga Sangha Forest Reserve, Central African Republic (CF).

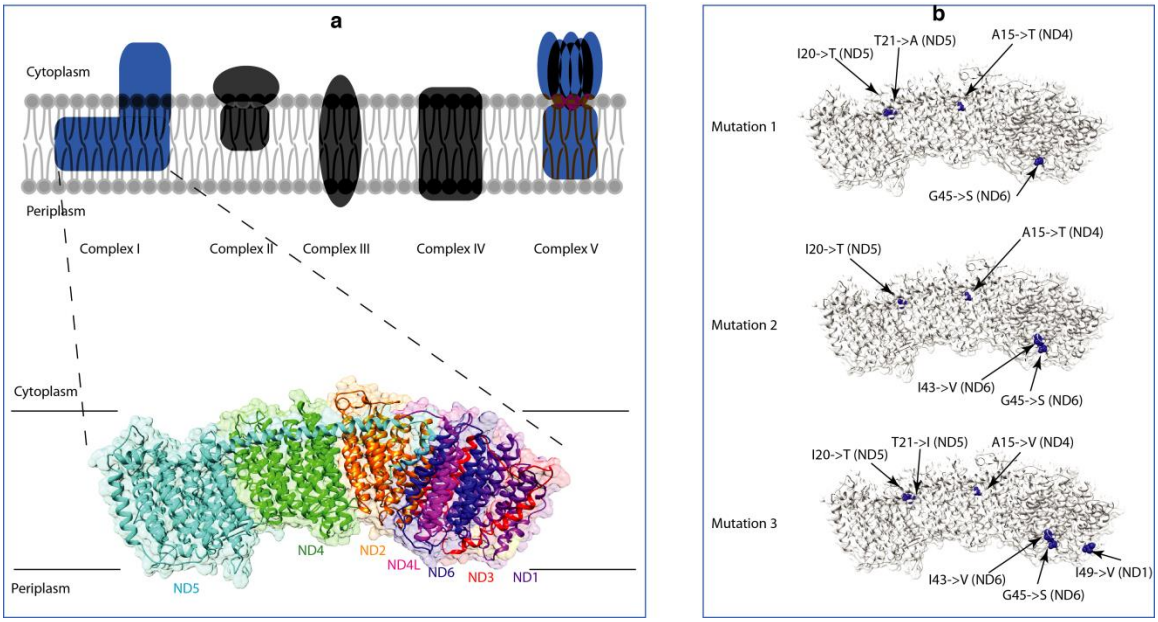


Figure 3: Our predicted models of the African elephant complex I. (a) Simplified drawing of the mammalian ETC with the five complexes that are involved in the OXPHOS pathway. These complexes are located on the inner mitochondrial membrane. The enlarged image shows the predicted African elephant protein structure for the mitochondrial DNA encoded genes of complex I. Chains are represented by different colors (dark purple = ND1, orange = ND2, red = ND3, green = ND4, light purple = ND4L, light blue = ND5, dark blue = ND6). (b) The three different forest elephant mutation models. Selected amino acid substitutions are mapped onto the savanna elephant predicted structure and are shown in blue. The Mutation 1 model represents SL, mutation 2 represents CI and GA, and mutation 3 represents CF. The mutations are labeled based on their chain ID, and with the savanna elephant residue listed before the altered forest elephant residue.

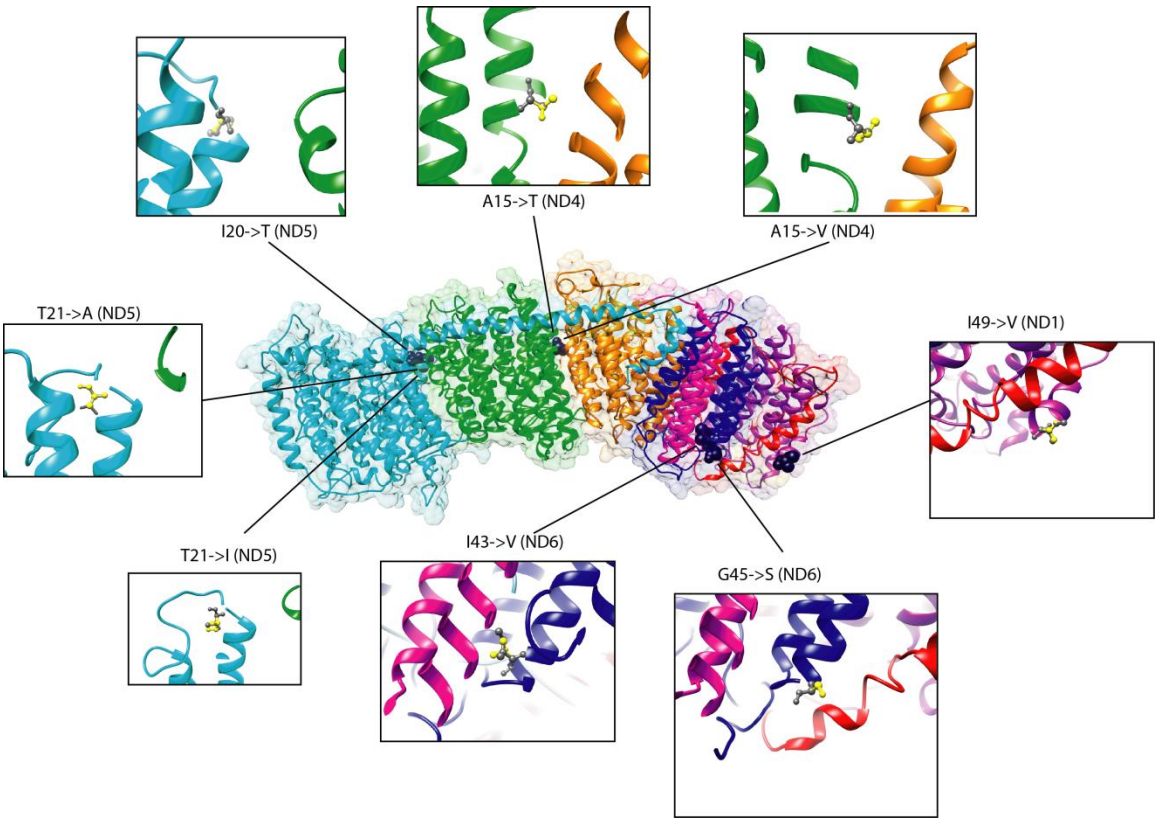


Figure 4: The atomic level structure for each of the selected amino acid substitutions as shown on our predicted model for the African elephant complex I. Mutations are shown in blue. The enlarged images show the African savanna elephant amino acid side chain in grey and the African forest elephant amino acid side chain in yellow.

CHAPTER 3:
MODELING PHYSIOLOGICAL FACTORS THAT INFLUENCE CROP RAIDING BEHAVIOR IN THE AFRICAN SAVANNA ELEPHANT

Abstract

Crop raiding is an increasing problem in areas where human and elephant population intersect. Traditional deterrent methods to crop raiding behavior are short-term solutions to which elephants frequently habituate over time. In order to develop long-term solutions for this problem, we need to understand the factors that influence this behavior in individuals. We collected dung samples from crop raiding and non-crop raiding African elephants in southern Kenya, including Maasai Mara National Reserve, and used genetic methods to identify and sex individuals, radioimmunoassay kits to measure glucocorticoid concentrations, and fecal flotations to quantify gastrointestinal parasite loads. We used generalized linear mixed modeling to assess the effects of age, sex, glucocorticoid metabolite levels, and parasite loads on crop raiding in elephants. After evaluating our models using the Akaike Information Criterion, we found that the highest ranked model included parasite load as the sole fixed effect, with crop raiders having lower parasite loads than non-crop raiders. The addition of demographic factors and glucocorticoid concentrations did not improve the model. This is the first study to confirm crop raiding by female elephants using molecular techniques. Our results suggest that crop raiding may provide fitness benefits in the form of lower helminth levels. This study emphasizes the need to learn more about the physiology of individual

crop raiders to develop long-term mitigation strategies for this form of human-elephant conflict.

Introduction

As the human population grows and expands into natural habitat, human-wildlife conflict is an ever-increasing issue facing the conservation of species worldwide. This is especially true for large mammals, such as the African elephant (*Loxodonta africana*) (Naughton-Treves 1998). With an estimated 70% of the range of the African elephant outside protected areas (Blanc 2007), human-elephant conflict is inevitable and the number of reported events is on the rise (Hoare and Du Toit 1999, Sitati, Walpole et al. 2003). Human-elephant conflict occurs where human and elephant populations intersect, and one of the most common and destructive forms involves elephants feeding or otherwise damaging agricultural areas, a behavior referred to as crop raiding. Crop raiding is a high-risk behavior that can result in both human and elephant fatalities when farmers actively guard their crops from such conflict (Wakoli and Sitati 2012). This makes efforts to conserve the elephant more difficult because it has a negative influence on peoples' attitudes towards the animals (Kioko 2006).

Studies on crop raiding have largely focused on spatial and demographic patterns of individuals participating in this risky behavior (Hoare 1999, Graham, Notter et al. 2010, Chiyo, Moss et al. 2012). Crop raiding is known to occur most frequently at night when there is a lower probability of detection by humans (Gunn, Hawkins et al.

2013) and in settlements with closer proximity to elephant habitat or refuge areas (Guerbois, Chapanda et al. 2012). Recent studies that have employed molecular sexing techniques found that all African elephant crop raiding was done by males and that habitual crop raiding individuals contribute to a greater proportion of raiding events (Ahlering, Millspaugh et al. 2011, Chiyo, Moss et al. 2011). Based on observational data, Sitati et al. (2003), however, report 68% of crop raiding incidences involved female-led groups.

Much attention has been given to identifying and measuring the effectiveness of deterrent methods. Traditionally, farmers have used multiple tactics that involve actively guarding their crops, scaring off animals using drums or firecrackers, building barriers, and planting unsavory crops along the perimeter of their farms (Osborn and Parker 2003). Most recently, beehive fences have proven successful in preventing African elephants from crop raiding (King, Douglas-Hamilton et al. 2011). While developing deterrent methods is useful in the short-term, elephants are highly intelligent and often become habituated to such actions, rendering them ineffective over time (Taylor 1999). To produce long-term solutions to crop raiding, it will be necessary to understand the mechanisms driving this behavior.

Few studies have approached this problem by studying the physical condition of individuals that crop raid. Ahlering et al. (2011) analyzed fecal glucocorticoid metabolite (FGM) concentrations in crop raiding elephants, and found male crop raiders had higher

FGM values than non-crop raiders, suggesting stress plays a role in this behavior. Another study estimated elephant body size based on hind foot measurements and discovered that adult male crop raiders were larger than their non-raider counterparts (Chiyo, Lee et al. 2011). They suggest the high-risk, high-reward of having a larger body size allows crop raiding males a competitive edge in mating success.

Given the risks involved in this behavior, we investigated possible physiological benefits to elephants that crop raid. Both high gastrointestinal (GI) parasite infection and FGM concentrations have been shown to depress the vertebrate immune response (Gause, Urban Jr et al. 2003, Bourgeon and Raclot 2006). Studies of the relationship between glucocorticoid hormones (i.e. stress hormones) and parasites in free-ranging wildlife, however, have produced inconsistent results. Some found support for an association between lower FGM levels and lower parasite loads (Raouf, Smith et al. 2006, Pedersen and Greives 2008), while others found no such relationship (Goldstein, Millspaugh et al. 2005, Monello, Millspaugh et al. 2010). In an anecdotal observation, one study on noninvasive stress and reproductive measures in free-ranging African elephants found large numbers of nematodes in the feces of individuals with the highest levels of cortisol metabolites (Foley, Papageorge et al. 2001).

In this study, we investigated the combined effect of parasite loads and FGM levels in elephants that partake in crop raiding. Our objectives were: (1) to gain demographic data on the crop raiding individuals through field and molecular

techniques, and (2) to model physiological factors that may be influencing crop raiding behavior in the African elephant. Gaining a deeper understanding of the biological processes that may be driving crop raiding events is important for developing long-term solutions to this inter-species conflict.

Materials and Methods

Study Area

The adjacent Transmara and Narok Districts are located in south-west Kenya along the border of Tanzania and include the protected lands of Maasai Mara National Reserve (MMNR) (Figure 5). The western-lying Transmara District spans an area of 2900 km^2 that is separated from the protected area of MMNR by a steep escarpment. Approximately 2200 km^2 of this region is inhabited by people. Annual rainfall averages 1200-1500 mm and occurs during two main wet periods from March to June and from November to December. The landscape is made up of Afro-montane, semi-deciduous, and dry-deciduous forests and *Acacia* savanna woodlands. East of Transmara District is Narok District, which comprises a much larger area of approximately 6,000 km^2. Narok District can be divided into three regions that have biogeographical and climatic differences: the protected MMNR, the Siana Plains and the Loita Plains. This study was conducted in the northeastern division called the Loita Plains that are characterized by dwarf shrub and whistling thorn (*Acacia drepanolobium*) grasslands. This region has an average annual rainfall of 700mm that follows the same bimodal pattern as Transmara District.

The historical inhabitants of both districts are the Maasai people who have traditionally lived as pastoralists. In recent times, however, both districts have undergone rapid changes in land use, and much of the unprotected area has been converted to agricultural lands that largely produce maize, wheat and sugar cane. Increasingly, the remaining forested areas are being slashed and burned for fuel sources. As cultivation and habitat loss have increased, so have incidences of crop raiding by elephants and other species (Sitati, Walpole et al. 2003, Sitati, Walpole et al. 2005).

Sample Collection

Samples were collected from both districts during the first sampling session from May-July 2011 and a second sampling session in Transmara District from November-December 2011. Both sampling sessions were timed to occur during the wet season where there is a high incidence of crop raiding events (Benedix, Becker et al. 2009); peak crop raiding occurrences correspond to the harvest periods when the crops are ripe (Sitati and Walpole 2006). We did not collect from Narok District in the second session, because the farmers there only harvest their crops once per year. Hence, there were no raiding incidents to collect samples from Narok during the second session. We obtained permission from the farmers to collect the samples from crop raiding events and labeled those collected directly from or adjacent to the damaged field as crop raiders.

Three samples were collected from each dung pile that was no more than 12 hours old. To estimate the age of the elephant, we averaged circumference measurements of up to three boli and compared our values according to Jachmann and Bell (1984). Age classification was based on Moss's (2001) definitions: 1-4 years as juveniles, 5-12 years as sub-adults, and individuals over 12 years as adults. For genetic analyses, approximately 20g of dung were collected from the slimy exterior of the dung boli. These samples were boiled in the collection tube to prevent the transportation of pathogens, then stored in Queens College preservation buffer (20% DMSO, 0.25 M EDTA, 100 mM Tris, pH 7.5, saturated with NaCl (Amos, Whitehead et al. 1992)). For hormone analysis, homogenized samples were collected by mixing 10g of dung from at least 3 boli whenever possible and stored in liquid nitrogen. Before samples were brought back to the University of Missouri (MU) on dry ice and transferred to a freezer, they were treated with 2% acetic acid to destroy pathogens (Millspaugh, Washburn et al. 2003). For parasite analysis, we homogenized samples by mixing 10g of dung from moist, inner regions of the boli. These samples were stored in a cool, dark place until being processed to sample gastrointestinal nematodes.

Genetic Methods and Analyses

Total genomic DNA was extracted from dung samples in a lab dedicated to noninvasive DNA extractions (Eggert, Maldonado et al. 2005) using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA) with modifications as described in Archie et al. (2006). To confirm dung samples came from unique individuals, we genotyped all

samples at 10 microsatellite loci (FH48R, FH60R, FH94R, LA6R, LAT13R, FH67, FH126, LA4, LA5, LAT05); (Comstock, Wasser et al. 2000, Eggert, Ramakrishnan et al. 2000, Comstock, Georgiadis et al. 2002, Archie, Moss et al. 2003, Eggert 2008). Loci were amplified in three multiplexes using the manufacturer's protocol of the Qiagen Multiplex PCR Kit (Qiagen, Valencia, CA), adjusted proportionally for 7.2 µL volumes, using an Eppendorf Mastercycler ep thermocycler (Eppendorf, Hauppauge, NY). The PCR profile included an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles of denaturation of 94°C for 30 seconds, primer annealing at either 58°C or 56.5°C for 90 seconds, and elongation for 72°C for 1 minute, ending with a final elongation step at 60°C for 30 minutes. A negative control sample was included with every PCR to detect contamination, as well as a positive control extracted from a captive savanna elephant to standardize allele scores. PCR products were separated in an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) at the MU's DNA Core Facility and allele sizes were scored by comparison with a LIZ600 size standard using GENEMARKER version 1.95 (Softgenetics, State College, PA).

To determine whether the loci have sufficient power to differentiate individuals, we performed a probability of identity ($P_{(ID)}$) test (Paetkau, Calvert et al. 1995) using GENALEX v. 6.41 (Peakall and Smouse 2005). We set our significance value for $P_{(ID)}$ at $p \leq 0.01$, within the suggested guidelines of Waits et al. (2001). Since allelic dropout is known to commonly occur in fecal DNA samples, we confirmed all heterozygous genotypes at least twice and all homozygous genotypes at least three times. If samples

did not amplify the minimum number of times at each locus or lacked reproducibility between runs, they were not included in this study. We used GENALEX to match samples and confirm unique genotypes, and used GIMLET to calculate the genotyping error rate across all loci (Valière 2002). In addition, we used the molecular sexing technique on each genotyped sample as described by Ahlering et al. (2011). Samples sexed as females were confirmed three times, and samples sexed as males were confirmed twice.

We tested for deviations from genotype frequencies expected under Hardy Weinberg equilibrium (HWE) and for linkage disequilibrium in GENEPOP v. 4.0.10 (Rousset 2008). For this, we divided our dataset into 3 groups: MMNR, Narok and Transmara. To decrease the chance of type I error (Rice 1989), we assessed the significance of results after Bonferroni corrections were applied We used MICRO-CHECKER v. 2.2.3 (Van Oosterhout, Hutchinson et al. 2003) to test for the presence of null alleles. To test for genetic differentiation between individuals sampled in MMNR, Narok, and Transmara Districts, we calculated pairwise F_{ST} values in ARLEQUIN v3.5 (Excoffier and Lischer 2010) and evaluated their significance using 10,000 permutations.

Hormone Methods

In the lab, samples were stored at -80˚C until being freeze-dried in a lyophilizer (Freeze-dry Specialties, Inc., Osseo, Minnesota) for 24-36 hours. Dried samples were ground and sifted through a stainless steel mesh to remove large debris. Approximately

0.2g of the feces was mixed with 2.0mL of 90% methanol and vortexed for 30 minutes at high speed. Samples were immediately centrifuged at ~1900*g* for 20 minutes, and the supernatant was stored at -20˚C until assayed. FGM concentrations were measured using a corticosterone I^{125} double-antibody RIA kit (Cat. #07120103, MP Biomedicals, Solon, Ohio) following methods validated for elephants (Wasser, Hunt et al. 2000). Inter-assay variation was 2.8% and average intra-assay variation was 2.0%.

Parasite Methods

Fecal samples were typically processed within 12 hours of sample collection. We used the Cornell-McMaster dilution egg count method to quantify GI nematode egg burden for each dung sample (Bowman 2008). Although the correlation between fecal egg counts and the number of adult parasites in the host can vary, egg counts provide a valuable noninvasive technique of assessing relative infection levels across hosts (Bryan and Kerr 1989, Stear, Bishop et al. 1995). A saturated sucrose solution with a specific gravity of 1.3 was used for all egg counts. We used a standard formula based on the known weight of feces (10g), the volume of water used (150mL), and the volume of the aliquots loaded into the counting chambers (0.30mL) to calculate eggs per gram of feces (EPG):

[(countchamber 1) + (countchamber 20)] x 50 = EPG

Statistical Analyses

Preliminary data analyses were conducted in the R statistical programming environment (R Development Core Team 2010). Both the FGM and EPG data exhibit non-normal probability distributions (Poisson). Nonparametric Wilcoxon signed rank tests were used to determine whether significant differences existed between crop raiders and non-crop raiders, and between the two sampling sessions.

The preliminary data analyses were used to inform the development of *a priori* models of factors influencing crop raiding behavior. Generalized linear mixed models using Laplace approximations were fit to the data using physiological and demographic variables using the lme4 package (Bates, Maechler et al. 2013). The model family was defined as "binomial," and a probit link function was used due to problems with model convergence when using the logit link function. Start values were determined by fitting generalized linear models to the models without random effects using the base stats package in R (R Development Core Team, 2010). Fixed effects of EPG and FGM values, sex (male or female), and age class (sub-adult or adult) were investigated for their explanatory power. Due to potential sampling effects and pseudoreplication by individual, random effects for individual elephants (denoted as subject) and sampling session (denoted as year) were included in all models. Model selection was completed by calculating Akaike information criterion (AIC) values and ranking models with $\Delta AIC_C \leq 2$ as the best fitting model(Burnham and Anderson 2002). A null model was established as a base for comparison of explanatory power.

Results

Genetic Analyses

The $P_{(ID)sibs}$ for all 10 loci was 5.2×10^{-4},and we determined we needed to genotype samples at a minimum of 6 loci ($P_{(ID)sibs} = 7.3 \times 10^{-3}$) in order to reliably identify individuals. Samples that were not genotyped at the minimum 6 loci were excluded from analyses. A genotyping error rate of 2.58% across all loci was observed.

Our complete dataset contains 101 samples. Of these, seven individuals were sampled twice (four between different seasons and three within the same season). Of those 94 individual samples, 41 were collected from crop raiding elephants and 53 were control samples (Table 7). Of the crop raiding samples, 26 were from females and 15 were from males. Of the non-crop raiders, 33 were female and 20 were male. Only sub-adults and adults were included in our analyses.

We found that one locus deviated from HWE after Bonferroni correction (FH48R: $p = 0.0003$) in Transmara but not in other locations. We did not detect significant linkage disequilibrium between any pairs of loci. Because the deviation from HWE occurred in only one location and did not affect our ability to identify individuals, we retained all loci in the study. Results from ARLEQUIN revealed no significant genetic differentiation between samples collected in MMNR, Narok and Transmara; thus, we classified our dataset as one genetic population.

Statistical Analyses

Crop raiding behavior was best explained by EPG values (Table 8a). The best fit model included parasite load as the only explanatory variable, with no other models falling within 2 AIC_C units. The next best fit models include EPG values and demographic factors. Our null model ranks higher than models based on FGM concentrations that do not also include EPG values. The slope of the best fit model shows a slight negative trend (Table 8b), which supports our conclusions of lower parasite loads in crop raiding elephants (Figure 6).

Results from the Wilcoxon signed rank tests show a significant difference between EPG values for crop raiders and non-crop raiders for the combined dataset from both sampling sessions. When data for each sampling session were analyzed separately, however, there was no significant difference between crop raiders and non-crop raiders (Figure 6). FGM concentrations did not differ significantly between crop raiders and non-crop raiders, supporting our modeling results (Figure 7). Given the small sample size of repeat captures, we were unable to test for any longitudinal effects of FGM and EPG values in individual animals.

Discussion

Our investigation of the demographic and physiological factors that may influence crop raiding behavior in the African elephant supports Sitati et al.'s (2003) findings that female groups frequently crop raid in this region. This is the first study to

document crop raiding by female elephants using molecular sexing methods. With female crop raiders typically being in the minority, most other research has focused on understanding why males participate in this risky behavior. Some hypothesize that male elephants crop raid to increase their nutritional intake in order to maximize reproductive success (Sukumar and Gadgil 1988, Hoare 1999). A recent study found larger body sizes in crop raiding males (Chiyo, Lee et al. 2011), lending support to this hypothesis.

Cultivated crops have higher palatability and nutritive value than natural forage, thus elephants are attracted to crops as a food source (Sukumar 1990). This feeding strategy is supported by optimal foraging theory, which predicts that individuals will attempt to maximize their net energy intake when feeding (MacArthur and Pianka 1966, Stephen and Krebs 1986). While the hypothesized trade-off between the risks and the nutritional gains involved in crop raiding may be readily applied to males, it may also be applied to females. Female African elephants live in family units of genetically related individuals that are led by the oldest female, the matriarch (Moss 2000, Archie, Moss et al. 2006). The matriarch is responsible for making choices that impact her family, including where to forage. The potential nutritional benefits associated with crop raiding combined with the highly fragmented elephant habitat in our study area may explain the high incidence of female crop raiders.

Our modeling results indicate that GI parasite load (EPG) is the best predictor of crop raiding behavior, with crop raiders having fewer parasites than non-crop raiders (Figure 6). In both wild and domesticated species, studies have found that host susceptibility to GI parasite infection is strongly influenced by nutrition (Coop and Kyriazakis 2001, Ezenwa 2004). For instance, a study of olive baboons (*Papio anubis*) found that wild-foraging troops had a significantly higher helminth parasite load than a crop raiding troop and suggest this may be explained by the crop raiding individuals having better body condition due to their nutritional intake, thus making them more capable of fighting off parasitic infection (Weyher, Ross et al. 2006). Improved nutrition, which elephants can achieve by eating crops instead of natural forage (Rode, Chiyo et al. 2006), increases host resistance to GI parasites (Abbott, Parkins et al. 1986, Wallace, Bairden et al. 1995). While the crop raiding elephants in our study have an overall lower GI parasite load, we were unable to determine parasite species richness, which may also be affected by crop raiding. Future studies that address this issue could provide further insight into what might be occurring with the GI parasite load by identifying possible inter-species transmission between elephants, domestic hoof stock and/or humans.

The inclusion of FGM levels did not improve our models of crop raiding behavior. Because we collected dung samples for our hormone assays, FGM levels represent an average over the approximate 30 hours before collection (Wasser, Hunt et al. 2000). Therefore, we were only able to measure stress levels leading up the crop raiding act, instead of gaining an estimate of stress while committing the raid. Nonetheless, FGM

measurements were not included in our top-ranking models. We sampled during seasons with high rainfall, which is associated with lower stress levels in elephants (Foley, Papageorge et al. 2001). Additionally, because the elephants in our study area inhabit a fragmented landscape that is interspersed with farmlands, they may be habituated to the presence of humans. Research on the physiological response of forest elephants (*Loxodonta cyclotis*) to human disturbance in Gabon found no significant difference in FGM concentrations between elephants sampled in around an oil field and those sampled in a protected national park (Munshi-South, Tchignoumba et al. 2008). The authors suggested that since elephants have been living in the disturbed landscape for decades, as is the case in our study area, they have likely habituated to the human-dominated landscape. Given that chronic stress (i.e. maintaining high levels of stress) can have negative physiological consequences, such as inhibition of immune function, reproduction or growth (Romero 2004), it would be to the benefit of individuals living in areas overlapping with human habitat to acclimate to such environments. We believe this might contribute to why we did not find a significant difference in FGM concentrations between crop raiders and non-crop raiders (Figure 6), in contrast to previous findings (Ahlering, Millspaugh et al. 2011).

Many statistical tools, such as the Wilcoxon rank signed test, only allow for the analysis of a single dependent variable, whereas biological models allow researchers to compare multiple observed variables (Hobbs and Hilborn 2006). Although traditional statistical testing did not yield significant results, the use of GLMM to account for the

ecological complexity of our study system allowed us to construct a biologically informative model that provides deeper insight into crop raiding behavior in the African elephant. Research on crop raiding, and human-elephant conflict as a whole, would benefit by analyses using a model testing approach to tease apart the intricate relationships of relevant factors.

Human-wildlife conflict is an ever-increasing threat to species conservation across the globe. Human-elephant conflict is particularly perilous as head-to-head confrontations can lead to death for both the human(s) and elephant(s) involved. Some argue that because of their intelligence and by adapting their behavior to raid largely at night, elephants perceive the risk involved in the activity (Ahlering, Millspaugh et al. 2011, Chiyo, Lee et al. 2011). Our results suggest that both male and female crop raiders may be in better physiological condition as a result of the better nutrition that comes with eating crops. Future work would benefit from a longitudinal analysis; while we captured repeat crop-raiders, our sample size was very small. We predict that the relationship between crop raiding and lower parasite levels will be more pronounced in habitual crop raiders.

This study highlights the need to consider the physical condition of the individual animal as we attempt to devise long-term solutions to crop raiding. Developing mitigation strategies for human-elephant conflict is a difficult and complicated task. However, it is important to know which elephants crop raid and what might be driving

this behavior in order to develop long-term solutions. If this high-risk foraging strategy confers some fitness benefits to the individual, we need to consider this information when creating deterrent methods.

Table 7: Demographic distribution of samples representing individuals collected in each sampling session (n = 94).

	Crop Raiders	
	Female	Male
Year 1	21	12
Year 2	5	2
	Non-Crop Raiders	
Year 1	13	9
Year 2	20	11

Table 8: (a) The difference in Akaike information criterion (ΔAIC) and the number of parameters (K) for tested models explaining crop raiding behavior (n = 101); model parameters include parasite (EPG values), hormone (FGM concentrations), sex (male/female), and age (sub-adult/adult).

Model	K	ΔAIC
Parasite + (1 | Subject) + (1 | Year)	3	0
Parasite * Sex + (1 | Subject) + (1 | Year)	5	3.09
Parasite + Sex + Age + (1 | Subject) + (1 | Year)	7	3.18
Parasite * Age + (1 | Subject) + (1 | Year)	5	3.69
Parasite + Sex * Age + (1 | Subject) + (1 | Year)	7	5.10
Parasite + Hormone + Sex + Age + (1 | Subject) + (1 | Year)	8	5.16
Null: 1 + (1 | Subject) + (1 | Year)	2	7.11
Hormone + (1 | Subject) + (1 | Year)	3	9.09
Hormone * Age + (1 | Subject) + (1 | Year)	5	12.42
Hormone + Sex + Age + (1 | Subject) + (1 | Year)	7	12.67
Hormone * Sex + (1 | Subject) + (1 | Year)	5	12.79
Hormone + Sex * Age + (1 | Subject) + (1 | Year)	7	14.12

(b) Estimated coefficients for the top ranked model; model parameter includes parasite (EPG values).

	Estimate	**SE**	**Pr(>|z|)**
Intercept	0.19189	0.36177	0.59582
Parasite	-3.6×10^{-4}	1.3×10^{-4}	5.3×10^{-4}

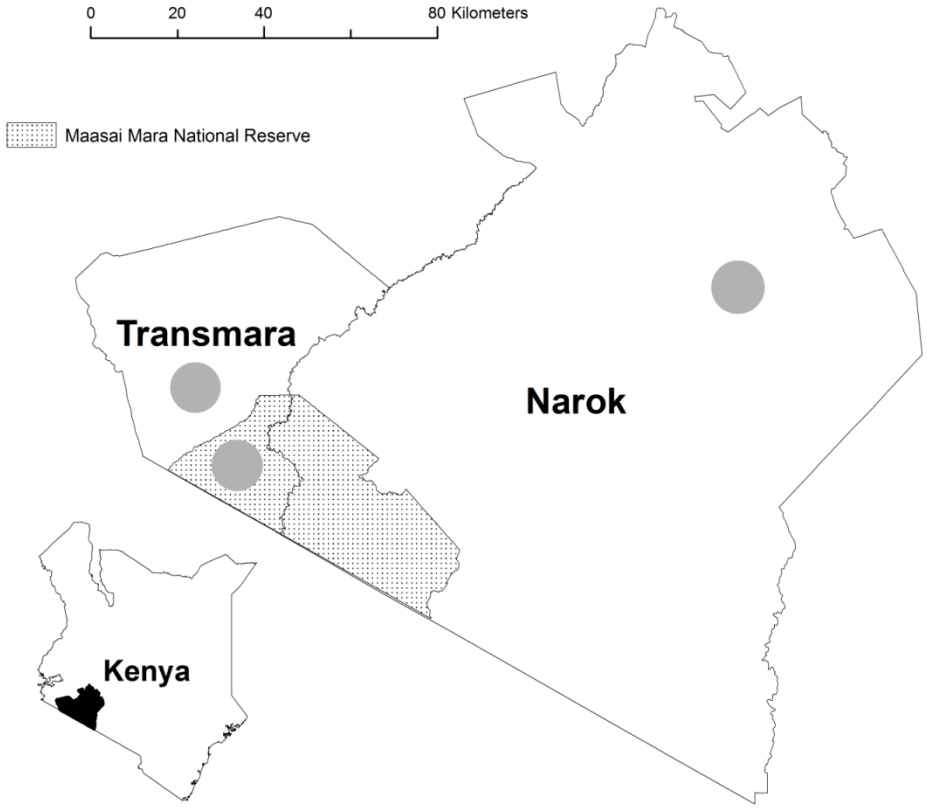
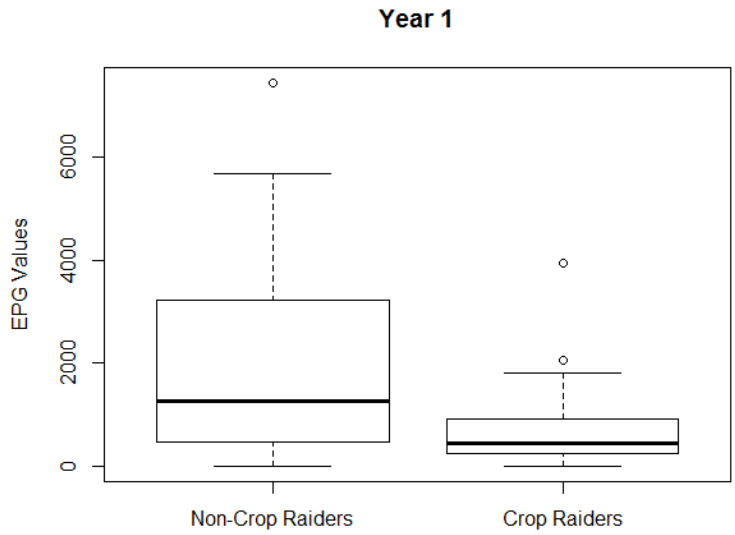


Figure 5: Map of Transmara and Narok Districts where samples were collected for this study. The area with hash marks indicates the borders of Maasai Mara National Reserve, and the grey circles are our sampling locations.

(a)



(b)

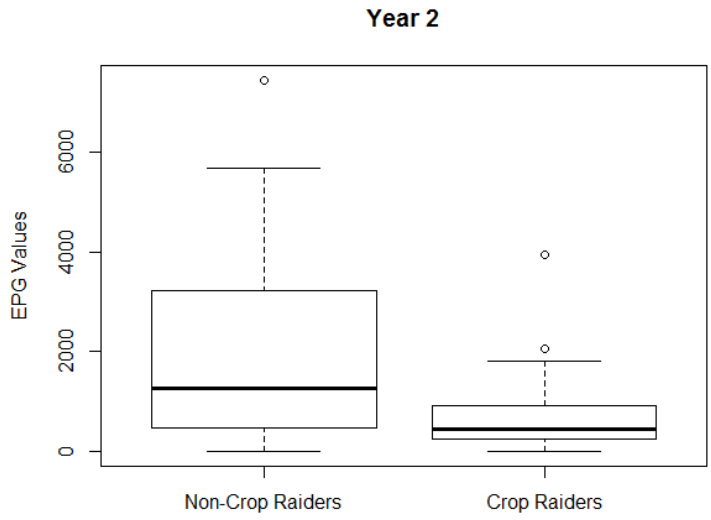


Figure 6: EPG values for crop raiders and non-crop raiders segregated by year. The black line represents the median value, the lines forming the box represent the first and third quartiles, and the whiskers extend to the minimum and maximum values. (a) Values for year 1 (b) Values for year 2.

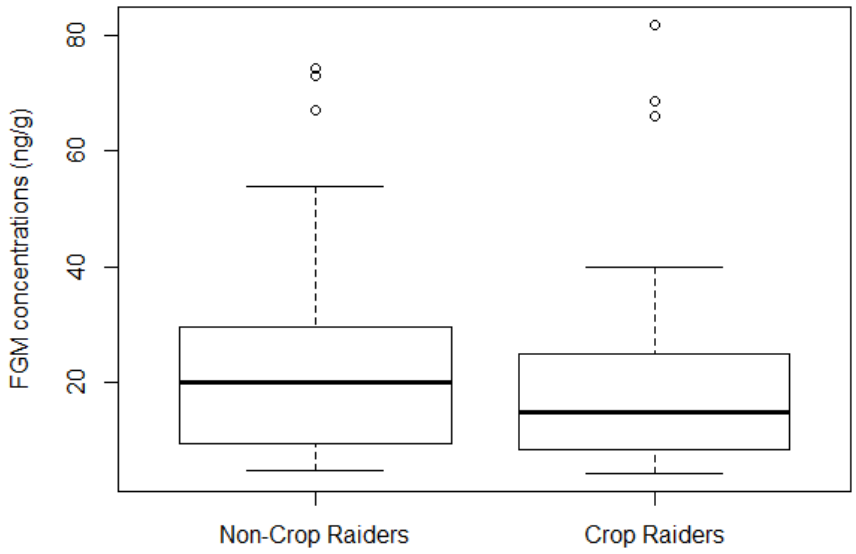


Figure 7: FGM concentrations (ng/g) between crop raiders (n = 45) and non-crop raiders (n = 61). The black line represents the median value, the lines forming the box represent the first and third quartiles, and the whiskers extend to the minimum and maximum values.

CHAPTER 4:
A COMPARISON OF THE FECAL MICROBIOTA OF THE AFRICAN FOREST AND SAVANNA ELEPHANT

Abstract

Vertebrates harbor gut microbial symbionts that serve many functions, one of which is to enhance metabolic capabilities. Factors such as host phylogeny, diet and behavior have been shown in many species to play a role in shaping the gut community composition. Our objective was to test and compare the effects of these variables on the microbiota in the African forest (*Loxodonta cyclotis*) and savanna (*L. africana*) elephant. We used pyrosequencing to analyze a region of the 16S ribosomal RNA gene to assess the fecal microbiota of 44 individual elephants. Our results revealed significant differences in the abundance of three bacterial phyla (Firmicutes, Bacteroidetes, and Proteobacteria) between the two species, while there were no significant differences within the *L. africana* individuals having habitat and behavioral modifications. These results suggest the influence of host phylogeny on the microbiota may be related to the nutritional requirements of each species.

Introduction

The vertebrate gut is host to trillions of microorganisms collectively known as the microbiota, whose cells outnumber that of the host by at least a factor of 10 (Savage 1977). Many of the microorganisms that reside in the vertebrate gut form a symbiotic relationship with their host and aid in nutrient uptake, contribute to energy

requirements, and help with the digestion of materials that would be otherwise impossible (Stevens and Hume 1998). The acquisition of symbiotic gut microbes has played a key role in the evolution of herbivory in mammals as they assist their hosts in the digestion of plant material by breaking down complex polysaccharides (Ley, Hamady et al. 2008).

While researchers have estimated the abundance of microorganisms through environmental sampling of soil, air and water, they have only been able to identify about 1% of the species that make up these communities since most cannot be cultured in the laboratory (Amann, Ludwig et al. 1995). Culture-independent techniques have since been developed to detect the richness of the gut microbiota. To date, the most robust technique has involved sequencing the prokaryotic 16S rRNA gene (Tringe, von Mering et al. 2005). This gene is 1,542 bp long, present in effectively all prokaryotes, and is conserved enough for accurate alignment, yet variable enough for phylogenetic analyses (Pace 1997). Recent advances in high-throughput sequencing technology have helped researchers explore the diversity and function of microbiota from a variety of environments, such as seawater, soil and fecal matter (Tringe, von Mering et al. 2005, von Mering, Hugenholtz et al. 2007, Ley, Hamady et al. 2008). Traditional methods of sequencing 16S rRNA involve amplification using the polymerase chain reaction (PCR), cloning, and Sanger sequencing, which are time and labor intensive, and thus limit the number of prokaryotes that can be discovered. With next-generation sequencing platforms, such as Illumina and 454, scientists can expand their dataset by orders of

magnitude and at costs that are increasingly becoming more affordable. These advances in sequencing technology allow biologists to ask questions on a much larger scale than was previously attainable. However, there is a still a great deal of work needed to understand the diversity and function of the microbial world (Rappé and Giovannoni 2003).

Diet is considered one of the main factors that influences the diversity of gut microbiota (Bäckhed, Ley et al. 2005). Given the important role the microbiota plays in digestion, it is no surprise that the community composition has been shown to vary with diet (Fernando, Purvis et al. 2010, Kau, Ahern et al. 2011). This relationship between diet and the microbiota has been shown to be significant on many levels. Ley et al. (Ley, Hamady et al. 2008) found that bacterial communities codiversified with their hosts based on a study of humans and 59 mammal species. Their results revealed that diet and phylogeny influence the microbiota and showed significant differences in the bacterial communities between herbivores, carnivores and omnivores. Other research on the human gut microbiota revealed that populations in Europe and Africa harbor unique groups of bacteria that help maximize energy intake (De Filippo, Cavalieri et al. 2010). A recent study on amphibians found that the microbiota differ greatly between tadpoles and adults, and attribute this variation to changes in dietary habits between life stages (Kohl, Cary et al. 2013). Much of our understanding of the influence of diet on the gut microbiota comes from controlled experiments on humans (Turnbaugh, Hamady

et al. 2009) and other model species (Murphy, Cotter et al. 2010), while little research on the subject has been conducted on free-ranging wildlife species.

The two sister species of the African elephant make an ideal system in which to study the evolution of the gut microbiota in nature. African forest (*Loxodonta cyclotis*) and savanna elephants (*L. africana*) have adapted to both their local habitats and diets since their divergence approximately 5.5 million years ago (Roca, Georgiadis et al. 2001, Brandt, Ishida et al. 2012). While both are monogastric hindgut fermenters (Clemens and Maloiy 1982), African forest elephants are found in the tropical forest regions of West and Central Africa, and their diet consists largely of browse and especially fruits when available (Rode, Chiyo et al. 2006). In contrast, African savanna elephants are distributed in the savannas of eastern and southern Africa, and their diet that consists largely (60-95%) of grasses (Codron, Codron et al. 2011). In addition, some elephants have altered their diets by taking advantage of nearby agricultural fields. This behavior, termed crop raiding, occurs whenever an elephant or a group of elephants feed in agricultural areas. The number of reported incidences of crop raiding is on the rise as human populations are growing and natural habitat is being converted to agriculture, largely to farm cash crops (Sitati, Walpole et al. 2003). One study of savanna elephants found that crop raiders derived up to 38% of their daily forage from agricultural crops (Chiyo and Cochrane 2005).

Using the African elephant as our study system, we assessed the effects of inter- and intraspecific characteristics on fecal microbiota communities. Specifically, we tested for taxonomic differences by comparing the bacterial communities between *L. cyclotis* and *L. africana*. Within *L. africana*, we examined the influences of habitat and diet on the bacterial community composition. Because studies on fecal microbiota have largely been conducted on model systems in controlled environments, our study goal was to test whether taxonomy, habitat and diet influence fecal microbial communities in a free-ranging, wildlife species.

Methods

Sample Collection and DNA Isolation

Descriptions of the study area and methods of sample collection, age estimation and crop raiding classification have been previously described (Chapter 2). We analyzed 48 dung samples for this study. We collected *L. africana* samples from the Transmara and Narok districts in southwestern Kenya during one field season from May-July 2011. *Loxodonta cyclotis* samples were collected from March-May 2010 at Lopé National Park, Gabon (Schuttler 2012). All samples were collected during the wet season to control for seasonal variability.

To confirm that samples represented unique individuals, *L. africana* samples were genotyped using 10 microsatellite loci (Chapter 2); *L. cyclotis* samples were genotyped at 12 microsatellite loci (2012). We used ML-RELATE (Kalinowski, Wagner et

al. 2006) to obtain a maximum likelihood estimate of the coefficient of relatedness, *r*, for each pair of genotypes and selected samples from individuals with r < 0.25, with the only exception being cases in which sub-adult or adult males were sampled in different habitats from related females. Samples collected in Maasai Mara and Narok district were categorized as savanna habitat (Serneels and Lambin 2001), while samples collected in Transmara district were classified as forest habitat (Sitati, Walpole et al. 2003).

Polymerase Chain Reaction and Library Generation

DNA was extracted from all dung samples using a protocol that employed bead beating (Yu and Morrison 2004), modified to accommodate double the start material (0.50g). The V4 region (253 bp) of the 16S rRNA gene was amplified using PCR primers 515F and 806R (Caporaso, Kuczynski et al. 2010), designed by the team leading the Earth Microbiome Project (Gilbert, Meyer et al. 2010) to amplify a region recommended by Liu et al. (2005). We modified the primers to include the Illumina forward, reverse, and multiplex sequencing primers, adding a 6bp barcode on the reverse primer to allow for multiplexing of 48 samples, similar to the methods of Bartram et al. (2011). All custom reverse primers were synthesized using the TruGrade service to reduce the risk of oligo crosstalk and barcode misalignment during downstream applications (IDT, Coralville, IA). PCR amplifications were carried out in triplicate. The 50 ul reactions contained 1 X PCR gold buffer, 0.2 µM dNTPs, 0.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1.5 mM $MgCl_2$, 10 X BSA (New England Bioloabs, Ipswich, MA, USA), 0.4 µM forward

primer, 0.4 µM reverse primer, and 2 µL of DNA template. Reactions began with a denaturation step at 95°C for five minutes, followed by 35 cycles of 95°C for one minute, 50°C for one minute, and 72°C for one minute, and ending with a final extension of 72°C for 10 minutes. Amplification of products of the correct size was verified by visualization in a 2% agarose gel, and fragments were purified using the AxyPrep Mag PCR clean up kit (Axygen, Union City, CA). The triplicate PCR products were mixed in equimolar quantities after quantification using a Fragment Analyzer (Advanced Analytical, Ames, IA). Paired-end 250 nucleotide multiplex sequencing was performed for the resulting libraries on an Illumina MiSeq (Illumina, San Diego, CA) at the University of Missouri's DNA Core facility.

Data Analysis

We used the QIIME pipeline (version 1.6.0) (Caporaso, Kuczynski et al. 2010) to analyze our raw Illumina fastq files. Default parameters were used to demultiplex and quality-filter sequences that were then clustered into operational taxonomic units (OTUs) based on a sequence similarity threshold of 97% using UCLUST (Edgar 2010) within QIIME. Representative sequences from each OTU were taxonomically classified using a QIIME-based wrapper of the Ribosomal Database Project (RDP) classifier (Cole, Chai et al. 2005) and setting 0.80 confidence threshold for taxonomic assignment.

We assessed beta diversity between samples in UniFrac (Lozupone and Knight 2005), which calculates the distance, or dissimilarity, between each pair of samples

using UPGMA. Based on QIIME's default setting, we ran 10 jackknifing resamples to determine support for tree nodes. We used weighted UniFrac clustering to account for the relative abundance of different types of bacteria in our samples and principal components were computed from these distances and mapped onto Principal Coordinate Analysis (PCoA) plots.

In order to test for statistical significance (p-value < 0.05) between the taxonomic classification of sequences between *L. cylcotis* and *L. africana* samples, we arcsine square root transformed our percentage data and ran Student's t-tests on the most abundant phyla. We further tested for statistical significance of crop raiding behavior and habitat type on the *L. africana* samples by arcsine square root transforming the percentage data and analyzing it with an ANOVA using R (R Development Core Team, 2010).

Results

A total of 10,525,428 reads were produced through Illumina sequencing of the fecal contents of African elephant samples. After quality filtering, there was a range of 101,377-511,235 reads per sample, except for one individual that had only 30,869 reads. These sequences were classified into 515 OTUs based on 97% sequence identity. Four samples were excluded from downstream analyses (two did not form proper contigs from the paired-end reads and two did not meet QIIME's quality control standards),

thus giving us 44 samples representing African forest (n = 11) and savanna (n = 33) individuals (Table 9).

The gut microbial community showed marked differences between *L. cyclotis* and *L. africana* (Figure 8). Analysis of the relative abundances of the most dominant bacterial phyla (Table 10) revealed significant differences between percentages of phyla Firmicutes, Proteobcteria and Bacteroidetes found in African forest and savanna samples. On average, *L. cyclotis* harbors significantly more Proteobacteria (42.4%) than *L. africana* (14.3%), while *L. africana* has significantly more Firmicutes (45.7% vs. 16.4%) and Bacteroidetes (15.3% vs. 10.1%) than *L. cyclotis*. Only 1.6% of the sequences were unclassified in both species.

The fecal microbiota composition for the intraspecific comparisons for the African savanna elephant are less differentiated (Figure 9, Table 11). Results from the ANOVA reveal no significant differences between the most abundant bacterial phyla Firmicutes, Proteobacteria and Bacteroidetes when testing the effects of crop raiding behavior and habitat differentiation (Table 12).

Principal coordinate analysis of weighted UniFrac distances reveal clustering of *L. africana*, but not *L. cyclotis* samples (Figure 10) with the first and second principal coordinates accounting for 30.59% and 11.48% of the variation respectively. When looking at the more specific categorization of the African savanna elephant samples,

there is no clear differentiation between the groups (Figure 11). However, individuals from savanna habitats (as represented by the light blue triangles and yellow circles in Figure 11) appear to cluster more tightly than those from forest habitat. In addition, the *L. africana* samples that are located in the upper left quadrant with the *L. cyclotis* samples (Figures 10 and 11) were sampled in forest habitat.

Discussion

Our results suggest that the greatest difference found between the gut microbial communities for the individuals included this study is between the two elephant species (Figure 10). Studies on a wide array of taxa, including horses, ants and fish, found similar support for the clustering of gut microbiota in relation to host phylogeny (Yamano, Koike et al. 2008, Anderson, Russell et al. 2012, Sullam, Essinger et al. 2012). Ley et al.'s (2008) research comparing the gut microbiota between 60 mammalian species, including both wild and captive samples, found that species grouped together regardless of whether they came from the same geographic location. This work suggests host phylogeny plays a large role in shaping gut microbial community composition. In our study it is important to note, however, that we sampled only one *L. cyclotis* and *L. africana* population each and that future work should further test the effect of taxonomy by including replicates from multiple populations.

The bacterial communities from our intraspecific analysis of the African savanna elephant do not appear to significantly cluster based on habitat and crop raiding

behavior (Figure 11). While individuals sampled in both forest and savanna habitats overlap, a closer inspection reveals that both crop raiders and non-crop raiders from the savanna are more clumped together than samples found in a forest habitat. Given that all our *L. africana* samples are from one genetic population (Chapter 2), it would be interesting to see whether adding additional populations to the sampling would show a larger effect of habitat type on the beta diversity analyses. Other studies that aim to assess ecological factors that shape the microbiota within a particular species, typically do so by comparing multiple populations (De Filippo, Cavalieri et al. 2010, McKenzie, Bowers et al. 2011, Schwab, Cristescu et al. 2011). Therefore, it may be that the focus of our sampling effort was too narrow to detect significant differences within the African savanna elephant gut microbial communities. Additionally, crop raiding behavior itself does not appear to influence the gut microbial community. Given that the gut passage time of an elephant is approximately 30 hours (Wasser, Hunt et al. 2000) and that we collected the dung sample deposited during a raiding event, we sequenced the microbiota as it was before the individual ingested crops. Therefore, our goal was to detect any change in the microbial community that may have identified nutritional deficiencies that might have led to elephants partaking in crop raiding behavior. While not as significant as interpersonal variation, research shows that the intrapersonal gut community structure does fluctuate over time (Costello, Lauber et al. 2009), and one longitudinal study in humans found that dietary changes resulted in a shift in the composition in the microbiota (Ley, Turnbaugh et al. 2006). Hence, future work that

samples individual crop-raiding elephants over time may reveal variation in gut microbes.

Consistent with studies of other species (Barker, Gillett et al. , Eckburg, Bik et al. 2005, Jami and Mizrahi 2012), we found the dominant phyla in the African forest and savanna elephant to be Firmicutes, Bacteroidetes and Proteobacteria (Tremaroli and Bäckhed 2012), with the first phylum having Gram-positive bacteria and the latter two having Gram-negative bacteria. These phyla are present in significantly different abundances between the two species (Table 10). Most notable is the large number (42.4%) of Proteobacteria found in *L. cyclotis*; 32.4% of the sequences from this phylum are classified to the genus *Acinetobacter* as compared to 9.5% in *L. africana* samples. Members of this genus are known pathogens commonly found on spoiled meats and in hospitals (De Filippis, La Storia et al. 2013, Oberauner, Zachow et al. 2013), however, many species are known to fix nitrogen and occur in relatively high abundances in another mongastric hindgut fermenter, the horse (O'Donnell, Harris et al. 2013). Nitrogen, which plays a key role in protein metabolism, is considered the most limiting nutrient for African herbivores (Van Soest 1994). Non-ruminants, such as the elephant, lose nitrogen in their feces because they have high gut passage times and cannot make use of microbial protein (Foose 1982). African forest elephants are mainly browsers and have a diet rich in fruits (White, Tutin et al. 1993, Campos-Arceiz and Blake 2011) that are rich in lipids and proteins (Short 1981). African savanna elephants have been shown to alter their diet to maximize nitrogen intake by consuming more nutritious grasses

during the wet season and then favoring browse in the dry season when grasses are less nutritious (Cerling, Wittemyer et al. 2006, Codron, Codron et al. 2011). Thus, savanna elephants may have more flexibility and control over their diets due to seasonal habitat changes, whereas forest elephants may require more nitrogen-fixing gut bacteria to maximize their nutrient uptake.

Another interesting difference is the higher abundance of bacteria within the Prevotellaceae family (phylum Bacteroidetes) in African savanna elephants (1.3%) as compared to forest elephants (0.6%). In a human study, De Fillipo et al. (2010) compared the microbiota between African children with a diet high in fiber content to European children with a diet high in animal protein, fat and sugar, and low in fiber. They found members of the genus *Prevotella* exclusively in the African children and explain it as a result of their high fiber diet and their need to maximize energy extraction from plant polysaccharides. Zhang et al. (Zhang, DiBaise et al. 2009) found similar results when analyzing the microbiota in obese individuals, and also attributes the presence of *Prevotella* species to higher energy absorption. Given that African forest elephants are 35-40% smaller than their savanna counterparts (Morgan and Lee 2003), it may be more vital for savanna elephants to have a greater proportion of Prevotellaceae bacteria to maximize their energy intake.

Overall, there are similarities in the elephant gut microbial community to that of the horse, which we would expect since both are herbivorous hindgut fermenters.

Studies have found significant differences in the gut microbiota for horses to be influenced by both diet and phylogeny (Yamano, Koike et al. 2008, O'Donnell, Harris et al. 2013). While we found significant differences between *L. cyclotis* and *L. africana* samples, our analysis of different habitats and behavior within the African savanna elephant did not yield strikingly different gut microbial communities. By increasing our sample size and including individuals from a greater geographic distribution, we might be able to better understand how these variables have shaped the evolution of the microbiota in the elephant. Future work should also include samples from the third elephant species, the Asian elephant (*Elephas maximus*), to further elucidate the role host phylogeny has in influencing bacterial community composition. This work illustrates the challenges of replicating controlled laboratory experiments on model species in non-model, free-ranging wildlife. It is important to test what is observed in nature, however, to understand the broad applications of evolutionary patterns.

Table 9: Samples represented in this study (n = 44) based on categorical classification to test specific hypotheses.

	Male	Female
Loxodonta cyclotis	6	5
Loxodonta africana:		
Crop Raiders, Forest	6	4
Crop Raiders, Savanna	2	2
Non-crop Raiders, Forest	3	5
Non-crop Raiders, Savanna	6	5

Table 10: Percentage of major bacterial phyla detected in the feces of African forest (n = 11) and savanna (n = 33) elephants. P-values were calculated with a Student's t-test and significant values are in bold

	Loxodonta cyclotis	***Loxodonta africana***	**P-value**
Firmicutes	16.4	45.7	**0.000**
Proteobacteria	42.4	14.3	**0.010**
Bacteroidetes	10.1	15.3	**0.002**
Verrucomicrobia	6.7	4.0	0.578
Spirochaetes	0.9	1.2	0.233
Lentisphaerae	0.6	0.2	0.172

Table 11: Percentage of major bacterial phyla detected in the feces of *Loxodonta africana* (n = 33) based on our a priori categories.

	Non-crop Raider, Forest	Non-crop Raider, Savanna	Crop Raider, Forest	Crop Raider, Savanna
Firmicutes	43.2	50.0	47.8	32.7
Proteobacteria	19.6	10.8	14.1	11.2
Bacteroidetes	16.0	13.7	14.2	22.5
Verrucomicrobia	2.7	4.3	3.8	6.1
Spirochaetes	0.6	1.4	0.9	2.6
Lentisphaerae	0.2	0.3	0.2	0.4

Table 12: Results from ANOVA tests of the effects of crop raiding behavior and habitat differences on the most abundant bacterial phyla found in *Loxodonta africana* samples (n = 33).

	Firmicutes		**Proteobacteria**		**Bacteroidetes**	
	Crop Raiding	Habitat	Crop Raiding	Habitat	Crop Raiding	Habitat
F value	0.61	0.00	0.05	3.10	1.11	0.38
DF	1	1	1	1	1	1
Pr (>F)	0.44	0.96	0.83	0.09	0.30	0.54

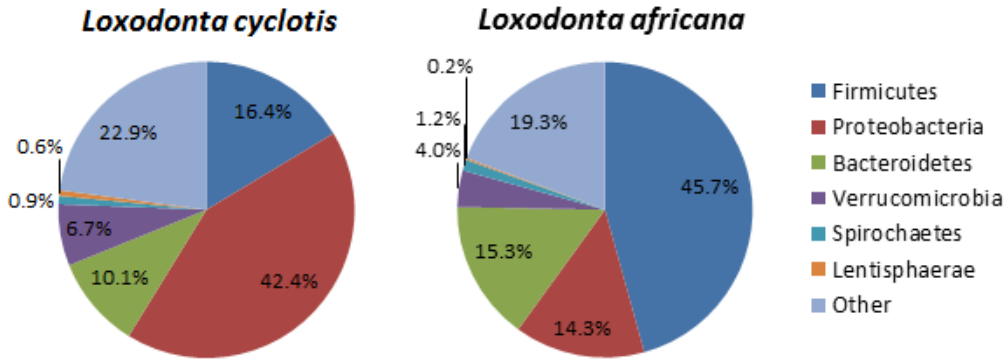


Figure 8: Taxonomic summary (presented as an averaged percentage) of the most abundant prokaryotic phyla classified from fecal samples of the African forest (n = 11) and savanna (n = 33) elephant.

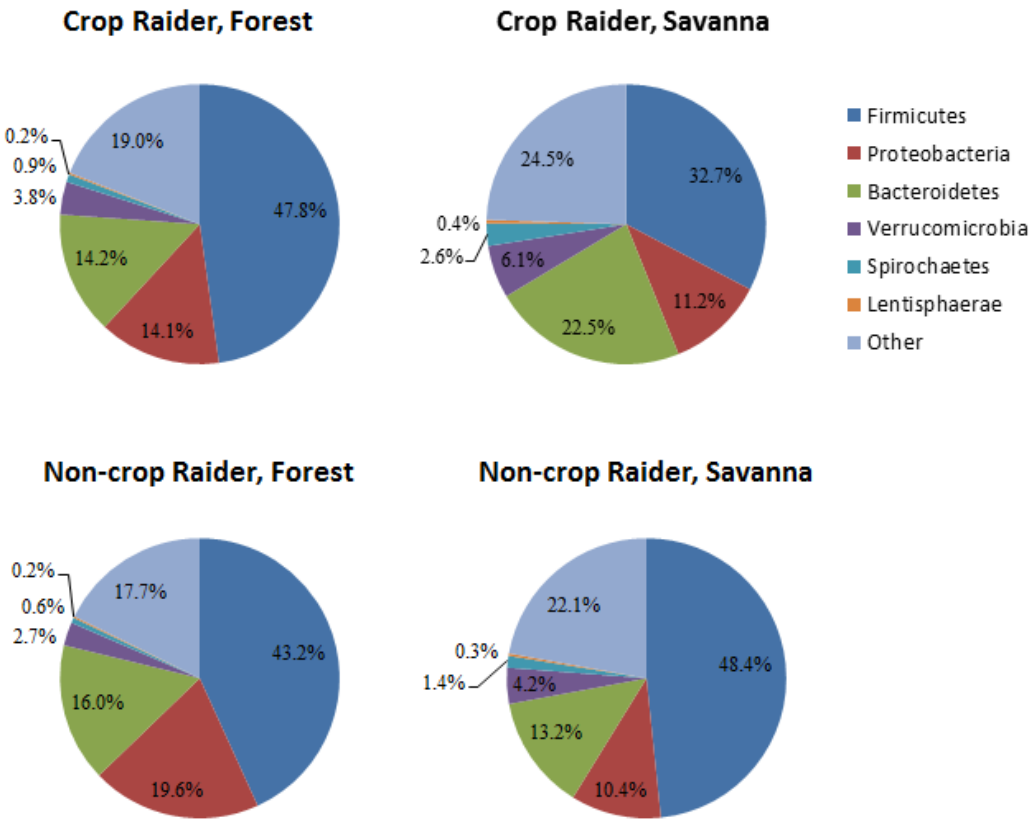


Figure 9: Taxonomic summary (presented as an averaged percentage) of the most abundant prokaryotic phyla classified from fecal samples of the African savanna elephant (n = 33). Pie charts represent samples categorized based on crop raiding behavior and habitat differentiation.

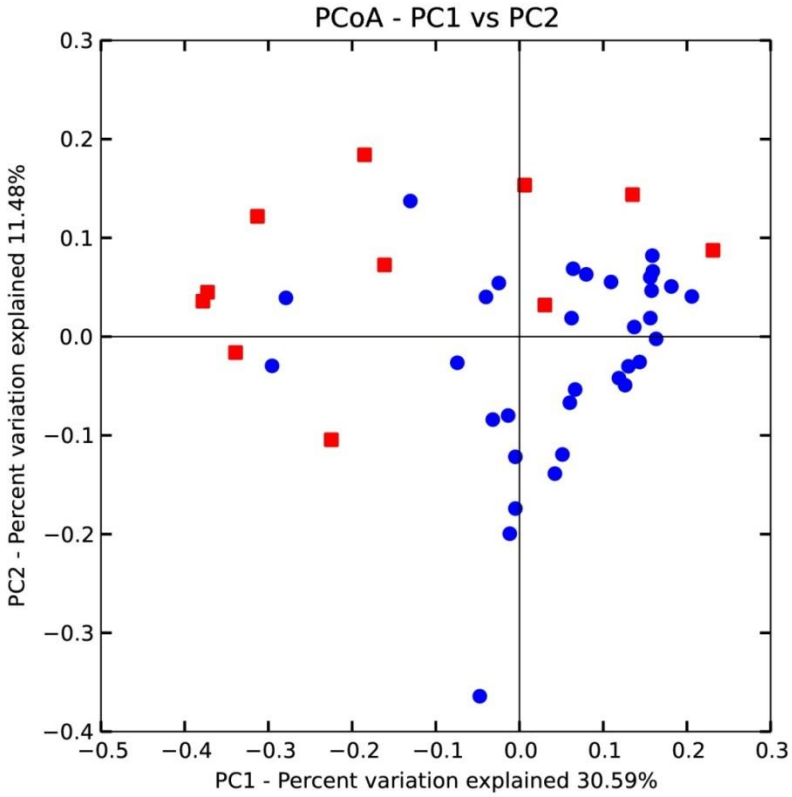


Figure 10: Principal Coordinate Analysis using weight UniFrac distances of the microbial communities from Loxodonta cyclotis (red squares, n = 11) and L. africana (blue circles, n = 33).

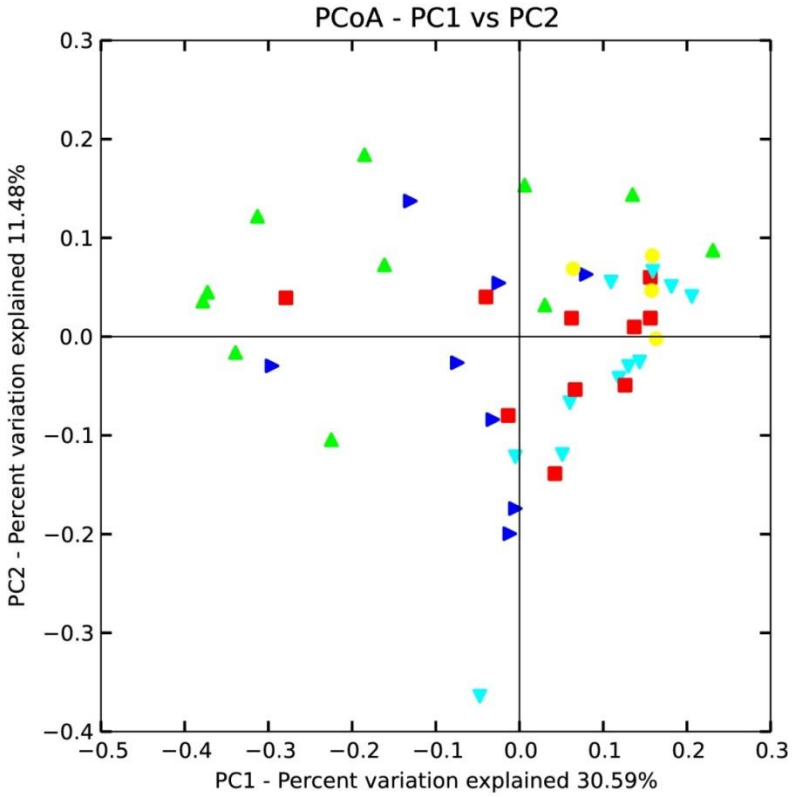


Figure 11: Principal Coordinate Analysis using weight UniFrac distances of the microbial communities from the African elephant samples included in this study. Green triangles represent Loxodonta cyclotis (n = 11) while the remaining data points are L. africana samples categorized as follows: red squares represent crop raiders, forest habitat (n = 10); dark blue triangles represent non-crop raiders, forest habitat (n = 8); light blue triangles represent non-crop raiders, savanna habitat (n = 11); and yellow circles represent crop raiders, savanna habitat (n = 4).

CHAPTER 5:
DISCUSSION

My research used noninvasive genetic sampling to answer questions relating to the ecology, evolution and behavior of the African forest and savanna elephant species. In my first study, I found evidence of positive selection in the mitochondrial DNA genes that code for complexes I and V of the oxidative phosphorylation pathway. This pathway produces the majority of ATP that drives cellular processes and is closely linked to metabolic activity. While the function of these enzymes is highly conserved given their vital role in energy production, I used an interdisciplinary approach to investigate the effect that selected residues might have on protein structure and function. My results suggest that these changes have contributed to the adaptation of forest and savanna elephants to their unique habitats. This study gives insight into links between molecular changes and adaptive phenotypic traits, which is one of the central foci of research in the field of molecular evolution. This work included a small number of samples, which limits the generalizability of the results over more finer-scale differences in habitat. Future research should include samples that span the geographic range of African forest and savanna elephants to identify associations between adaptive changes and ecological and morphological changes in these species. In addition, it will be important to identify and sequence the nuclear genes that code for the protein subunits that complete the enzymes involved in oxidative phosphorylation to better understand how the protein interactions might affect the function of this pathway.

My second study analyzed physiological factors that may influence crop raiding behavior, a highly destructive form of human-elephant conflict. The results of modeling the effects of stress hormone levels, demographic variables and parasite load indicated that parasite load alone was in the top ranking model. Interestingly enough, crop raiding individuals had lower parasite loads than their non-crop raiding counterparts, thus suggesting that there may be a fitness benefit to partaking in this risky behavior. Although previous work suggested that crop raiding males had higher stress levels than non-crop raiders, my results indicated that stress hormone levels did not improve the model. The population of elephants I sampled live in a highly fragmented habitat among human settlements and thus have likely habituated to the presence of humans. This work was the first known study to confirm crop raiding by females using molecular techniques. Given the fragmented landscape and the potential for higher fitness through lower parasitic infection levels, matriarchs may be leading their family groups to agricultural fields to eat the crops. Overall, this study emphasizes the need to learn more about the physiology of individual crop raiders to better understand the evolutionary underpinnings of crop raiding. An important extension of this work should involve a longitudinal analysis of serial crop raiders as compared to non-crop raiders to assess the long-term patterns of stress hormone levels and parasite loads in these individuals as they relate to this behavior. In order to develop long-term mitigation strategies to human-elephant, and human-wildlife, conflict, we need to identify the factors influencing this action in the problem animals.

Finally, I compared the fecal microbiota of the African forest and savanna elephant to gain insight into the influence of host phylogeny, diet and behavior on the community composition. After analyzing sequence data from the 16S ribosomal RNA gene for 44 individual samples, I found significant differences in the gut microbiota between these two species. Specifically, forest elephant samples were largely composed of bacteria from phylum Proteobacteria whereas in the savanna elephant, phylum Firmicutes was the most represented. These bacterial differences may be due in part to the unique diet of each species. While host phylogeny seemed to play an important role in shaping the gut flora, the intraspecific comparison of savanna elephant samples that were collected in varied habitats and in crop raiding incidents did not appear to be significantly different. However, all of the samples analyzed for this study came from one forest elephant and one savanna elephant population. Had I sampled more than one genetic population for each species, I might have been able to detect more fine-scale differences in the fecal microbiota that could be tied to habitat or behavioral differences. In order to further test the effect of taxonomy on the evolution of these communities, samples from the third extant elephant species, the Asian elephant (*Elephas maximus*), should be included.

Elephants are not only global symbols of conservation, they are also creatures that have cultural and historical significance. All of the extant elephant species live in increasingly fragmented areas due to human expansion, which frequently leads to

conflict. In order to develop effective management strategies for the survival of these species, we need to no more about their basic biology. My research provides insight into basic questions relating to the ecology, evolution and behavior of the two African elephant species. My research was conducted using noninvasive dung samples and replicated methods from similar studies using blood or tissue samples for genetic analyses, thus showing the breadth of what can be learned from dung. Additionally, the research questions I have focused on have traditionally been looked at using model species in controlled laboratory experiments. My results have added value by testing for similar relationships in free-ranging wildlife species.

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VITA

Tabitha Marie Finch was born on March 4, 1985 in Haverfordwest, Wales. She spent the first few years of her life living in Mississippi (where she survived Hurricane Elena), Tennessee, Pennsylvania and Wisconsin before settling in Tucson, Arizona. There, Tabitha graduated from University High School in 2003 and went on to attend Northern Arizona University in Flagstaff, Arizona for her undergraduate education where she earned a Bachelor of Science in Zoology in 2007. Tabitha was involved in several undergraduate research projects that included studying the effects of disturbance on Gunnison's prairie dogs, UV resistant genetic mutants of *Chlamydomonas monoica*, and comparing bird species diversity between different habitats. In addition, Tabitha spent a year abroad at the Universität Konstanz in Germany and completed a Bachelor of Arts in German from Northern Arizona University. After graduation, she traveled internationally and then worked as an intern with the US Army Corps of Engineers to record sea lion predation on salmon at the Bonneville Lock and Dam in Cascade Locks, Oregon. In fall 2008, Tabitha began her graduate work at the University of Missouri where she pursued her passion for conservation biology and studied the African elephant under the supervision of Dr. Lori Eggert. During this time she conducted two field seasons in Kenya outside Maasai Mara National Reserve to study crop raiding behavior. Additionally, while at the University of Missouri, Tabitha participated in numerous science outreach projects, including writing a weekly "Ask a Scientist" column for the Columbia Daily Tribune, a collaborative blog

on her research called “The Dung Diaries”, and organizing an after-school elementary science club. In October 2013, Tabitha moved to Birmingham, Alabama to begin working as the Manager of Science Education at the McWane Science Center. She defended her Doctor of Philosophy in Biological Sciences in November 2013 and graduated in December 2013.