CLOACAL MICROBIOTA OF CAPTIVE-BRED AND WILD ATTWATER’S
PRAIRIE CHICKEN, Tympanuchus cupido attwateri

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The Attwater’s prairie-chicken (*Tympanuchus cupido attwateri*; APC) is a species of grouse native to Texas coastal prairies and is on the critically endangered species list as a result of habitat destruction and overhunting. All of the current populations were captively bred and released into the wild. Survivorship for released APCs is very low, and individuals seldom survive to reproduce in the wild. One factor contributing to this may be an alteration in the gut microbiota as a result of captivity. Factors potentially influencing the gut microbial composition in captivity include antibiotic therapy, stress, and a predominantly commercially formulated diet. Recent studies have begun to shed light on the importance of the host microbial endosymbionts. Antibiotic administration, stress, diet, age, genotype and other factors have been shown to influence microbial populations in the gastrointestinal tracts of many different vertebrates. Sequencing of 16S rRNA gene amplicons on the Ion Torrent™ platform was used in this study to identify groups of bacteria in the cloacas as a surrogate for the gut microbiota in the APC. Antibiotic-treated and untreated birds, wild-hatched and captive-bred birds, and individuals sampled before and after release to the wild were examined. Significant differences were found between wild-hatched and captive raised birds both pre- and post release. In addition, there was extensive variation among the populations at the lower taxonomic ranks between individuals for each group of APCs. Principal coordinate analysis based on the weighted UniFrac distance metric further exhibited some clustering of individuals by treatment. These data suggest that captive breeding may have long-term effects on the cloacal microbiota of APCs with unknown consequences to their long-term health and survivorship.
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By

Stephanie E. Simon
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<td>APC</td>
<td>Attwater’s prairie-chicken</td>
</tr>
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<td>APCNWR</td>
<td>Attwater’s Prairie Chicken National Wildlife Refuge</td>
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<tr>
<td>ddNTPS</td>
<td>Di-deoxynucleosidetriphosphates</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleosidetriphosphates</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>ESS</td>
<td>Environmental shotgun sequencing</td>
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<td>FRWC</td>
<td>Fossil Rim Wildlife Center</td>
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<td>GIT</td>
<td>Gastrointestinal tract</td>
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<td>HMP</td>
<td>Human Microbiome Project</td>
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<td>iNKT</td>
<td>Invariant natural killer T</td>
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<td>NGS</td>
<td>Next-generation sequencing</td>
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<td>OTU</td>
<td>Operational taxonomic unit</td>
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<td>PCoA</td>
<td>Principal coordinate analysis</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PGM™</td>
<td>Personal Genome Machine™</td>
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<td>REV</td>
<td>Reticuloendotheliosis virus</td>
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<td>RDP</td>
<td>Ribosomal Database Project</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
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<tr>
<td>SCFA</td>
<td>Short-chain fatty acid</td>
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<td>Standard flowgram format</td>
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<td>Single nucleotide polymorphism</td>
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<td>WGS</td>
<td>Whole-genome shotgun</td>
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CHAPTER 1

INTRODUCTION

1.1 Attwater’s Prairie-Chicken

The population of Attwater’s prairie-chicken (Tympanuchus cupido attwateri; APC) was once abundant with a peak population approaching one million individuals 100-200 years ago and over 2.4 million hectares of prairie grassland habitat along the Gulf of Mexico; but, currently APCs number fewer than 200 individuals and are classified as a federally endangered species since 1967 [1]. The demise of the APC is attributed to natural factors, such as unfavorable weather, predation, and disease as well as artificial factors, including habitat loss and degradation, hunting [2] and more recently, possibly as a result of inbreeding [3].

Currently, nearly the entire population of APCs in the wild, consisting of less than 100 individuals, was either hatched in a captive-breeding facility or offspring from a released captive-bred hen. A captive population was developed in 1992 and now approximately 200 individuals exist in six captive-breeding facilities annually [1]. There are many factors related to the health and success of captive-bred APCs, such as avian reticuloendotheliosis virus (REV), immunocompetence, stress, and inbreeding, to name a few. Efforts are underway to identify all of the factors, including the study of the cloacal microbiota of captive-bred APCs described in this thesis, in order to improve survivorship.

1.2 Gut Microbiota

Recent studies have elucidated the roles of microbial endosymbionts to the health and development of their vertebrate hosts. The estimation that the number of genes in the human
microbiome outnumber human genes by 100 to 1 [4] highlights the importance of the host as an ecosystem. Currently a large collaborative project, the Human Microbiome Project (HMP), is underway to study the microbial ecology of every niche of the human being as a host. The goals of the HMP are to attempt to determine a “core microbiome,” elucidate host-microbe interactions, identify and characterize the various microbiota, and compare spatiotemporal differences, in order to understand the role of the human microbiome in human health and disease and to develop strategies for prevention and/or treatment [5]. In a similar manner, this project of the cloacal microbiota in captive-bred APCs applies the principles of the HMP to wild animal conservation.

The concept of a core microbiota refers to a shared collection of closely-related microorganisms typically associated within the same niche, such as the gastrointestinal tract or GIT [6]. While prokaryotes predated multicellular life on Earth for 2.5 billion years, multicellular and unicellular organisms developed a co-evolutionary relationship with, and in some cases dependence upon, their associated microbes. As expected there is a phylogenetic divergence exhibited between the vertebrate GIT habitat and niches like soils where free-living organisms preside. Phyla Firmicutes and Bacteroidetes are by far the most numerically dominant in the GIT habitats, while Proteobacteria have a large representation in free-living organism habitats [7]. Diet seems to be a primary predictor of a core microbiota of the GIT of healthy individuals. For example, there is less microbial diversity when comparing intraspecies samples, such as human fecal samples, than interspecies samples, such as samples from humans and the domestic cow [7]. Although a core microbiota of the gut may be established in humans, it is primarily seen at the phylum level as a great deal of variation at the lower taxonomic levels has
been demonstrated. The concept of a microbial genetic fingerprint comes into discussion when looking across individuals. While the same individuals may appear to have a similar microbial community profile at the phylum and class level, genetic variation can be seen at the species level. As one example of the tremendous amount of intraspecies diversity, one metagenomic study identified 10.3 million single nucleotide polymorphisms or SNPs in 101 genomes across 207 human individuals [8].

Health has increasingly been linked to symbiosis between the gut bacterial community and its host. Most interactions between animals and microorganisms do not result in disease. In fact, the normal gut microbiota in humans possess many vital metabolic capabilities that the host cannot otherwise perform, such as synthesis of essential vitamins and breakdown of host-indigestible plant polysaccharides and other plant products [9]. The host gut microbiota also affects a multitude of physiological properties, such as energy balance, pH, synthesis of vitamin K1 used in anabolic pathways, immune function, degradation of xenobiotics, and protection against pathogens [10, 11]. The species that make up these bacterial communities vary between hosts due to restricted migration of microorganisms, as well as host variability such as diet, genotype, age, gender, antibiotic administration, and colonization history [12, 13]. It has been shown that the intestinal microbiota is distinctly different between infant and adult [11], between healthy American and Chinese individuals [14], and between males and females at puberty and beyond [13].

Gut microbiota have further been credited in the development of the host immune system, modulation of the brain and nervous system, as well as potential causes of inflammatory bowel syndrome, asthma, and autoimmune disorders [15]. Studies between
germ-free mice and specific pathogen-free mice show a measurable difference in the amount of invariant natural killer T (iNKT) cells in the colonic lamina propria, and that the germ-free mice with accumulation of iNKT cells are susceptible to oxazolone-induced colitis and higher mortality [16]. Another immunology study found evidence of an immunomodulatory molecule produced by the ubiquitous gut microbe, *Bacteroides fragilis*, that helps to establish a proper balance between T helper 1 and T helper 2 cells, which is critical for healthy immunologic function [17]. Other results indicate that gut microbiota also affects the hypothalamic-pituitary-adrenal response to stress. For example, one group demonstrated germ-free mice with increased plasma levels of adrenocorticotropic hormone and corticosterone after acute restraint stress by placing the mice in a 50ml conical tube, while levels of brain-derived neurotrophic factor decreased, suggesting abnormal brain function [18]. Another group later observed that germ-free mice exhibited reduced fear and anxiety-like behavior, and found significantly increased levels of noradrenaline, dopamine, and serotonin in the striatum of the brain [19]. Small chain fatty acids or SCFAs, another type of signaling molecule, are products of bacterial metabolism and affect host immune response by altering the insulin-like growth factor-binding protein expression through histone deacetylase inhibition [20]. The presence of *Clostridium difficile*, an endospore-forming bacterium responsible for severe diarrhea following antibiotic therapy, has been associated with low concentrations of SCFAs [21]. Interestingly, the chicken gut microbiota produce higher concentrations of SCFAs than does the human gut microbiota and SCFAs appear to exhibit bacteriostatic activity against some pathogens [22], which may be beneficial to these birds.

One of the most dramatic factors affecting the composition of a healthy intestinal
microbiota is the use of broad-range antibiotics. Normal microbiota act as a barrier against the colonization of opportunistic pathogens as well as maintaining potentially harmful organisms, such as *C. difficile* and yeast, at low numbers [23]. Significant changes in the normal gut microbiota in healthy human volunteers have been documented following antibiotic treatments with ampicillin and amoxicillin. Such changes included an increase in ampicillin-resistant *Klebsiella* and *Enterobacter* strains with ampicillin, and detection of *C. difficile*, *Candida albicans*, and *Pseudomonas* species with amoxicillin, while there was an overall decrease in the numbers of commensal bacteria [23]. The most notable effects were observed under the administration of cephalosporins and clindamycin orally, where numbers of commensal enterobacteria and anaerobes decreased and incidences of toxin-positive *C. difficile* were detected in 7 of 10 patients post-clindamycin treatment and with 9 of 13 types of cephalosporins [23]. Often, the natural microbiota return a few weeks after short-term antibiotic administration by up to 88% similarity of pre-treatment composition; however, another study showed a shift in community members persisted for up to two years after 7-day treatment with clindamycin [24, 25]. A study performed on broiler chickens showed that 75-90% of the fecal samples at each time point were dominated by the phylum Firmicutes, but were significantly (*p < 0.05*) depleted after 7 and 14 days post-treatment with monensin/virginiamycin [26]. Clostridiales have been shown to be more active at the conclusion of antibiotic therapy, which may be attributed to their robustness and resistance to various antibiotics [27]. Relevant to the study discussed in this thesis, fluoroquinolones, which include the drug Baytril® or generic name enrofloxacin, have been shown to decrease the alpha diversity following therapy [21]. Notably, one example found a decrease in the abundance of
Faecalibacterium prausnitzii, which has been reported to exhibit anti-inflammatory activity and lower numbers of this bacterium have been associated with Crohn’s disease [21].

The studies of the gut microbiota as outlined in this introduction are used as a model for the gut microbiota in APCs. Even though the intestine is not being sampled directly in this study, cloacal samples have been shown to partially represent the gut microbiota in birds.

1.3 Metagenomics and Next-Generation Sequencing

The field of metagenomics has emerged recently due to technological advances in next-generation sequencing (NGS) and computing power to handle the enormous datasets. This application of modern genomic techniques to the study of microbial communities directly in their natural environments, bypassing the need for isolation and laboratory cultivation of individual species, is expanding our understanding of community ecology in microbes such as in the GIT. For community diversity studies, 16S ribosomal DNA sequencing has been the gold standard, but recently whole-genome shotgun (WGS) sequencing has emerged as an alternative strategy for assessing microbial diversity [28]. WGS sequencing has traditionally been applied to determining the complete genome sequence of one organism, but lately has been applied to capture sequences from many organisms [29].

Historically, 16S rDNA-based studies have been predominantly used for determining abundance of microbial populations in an environmental sample. This method is useful since the 16S ribosomal RNA gene exists in all prokaryotes known to date and represents at least 80% of total bacterial RNA. In addition, the 16S rRNA gene contains alternating regions of conserved and variable sequences, which is suitable for PCR amplification in that the primers are designed
to hybridize to conserved regions and the variable regions are replicated. Sequencing of these amplicons and comparing to known bacterial genomes has enabled investigators to identify organisms that could not otherwise be determined via biochemical or cultivation methods [28], as well as organisms that are underrepresented in particular environments. Subsequently, this method has contributed to the phylogenetic taxonomic classification system for Bacteria that exists today. However, 16S-based analysis has limitations and has thus propelled the advancement of WGS and environmental shotgun sequencing (ESS). For example, the 16S gene is limited to prokaryotes and thus viral and eukaryotic residents of particular environments are omitted from such community profiles [30]. Also, amplification and cloning bias is inherent in 16S rRNA protocols [31], sometimes as a result of suboptimal thermal cycling conditions and/or primer design. Using suboptimal primers may lead to an underestimation of evolutionarily distant members of a population since even the conserved regions undergo change over time, as well as under-representation of a single species or whole groups [32] if the primers are biased to the more common organisms. Some bacteria experience mosaicism and can tolerate the transfer of part or complete rRNA genes [33], which would affect the phylogenetic analysis of results if a distinct species is paired with another species sharing the rRNA gene. And finally, the copy number of rRNA operons may vary by as much as 15 [34], which would impact abundance estimates using this method.

Unfortunately, despite technological advances, there is a surprising amount of diversity between studies of the same environment. This is most likely due to the fact that culture-independent studies introduce inherent biases between the various methods for DNA extraction, PCR protocols, and 16S rRNA primers [35, 36]. The diversity within the bacterial cell
wall and/or cell membrane makes it difficult to design a universal disruption method and lysis buffer. Misrepresentations from the PCR process may be due to error-prone DNA polymerases as well as chimera formation due to amplification of DNA from multiple sources [37]. NGS has afforded many benefits to the field of microbial ecology; however, this technology also has limitations such as the inability to sequence the full-length (approximately 1,465bp) 16S rRNA gene, since the longer the fragment the greater the accuracy of microbial classification. With NGS, one or more of the nine variable regions of the 16S gene are targeted, but no single region has been universally accepted as the standard for 16S sequencing [37]. As stated, longer read lengths improve bacterial identification, and NGS technology is beginning to overcome such limitations by upgrading their platforms to at least 400bp at the present time. The sequencing process alone may cause misrepresentations of the relative abundances of microbial populations as a result of erroneous base calling [37]. However, the sequencing platforms typically have a quality control check and the downstream approaches using bioinformatics programs help to reduce sequencing errors. Although NGS has disadvantages, the advantages by far outweigh the limitations and therefore this technology was used in this project to identify the diversity in the cloacal microbiota of the APCs.

The next-generation sequencing platform used for this project was the Ion Personal Genome Machine®, or PGM™ by Ion Torrent™. In some cases, traditional Sanger sequencing was also employed. The Ion Torrent™ platform uses a sequencing-by-synthesis approach where flows of individual nucleotides are passed over a semiconductor chip containing millions of microwells that capture chemical information in the form of released protons and translates it to digital information in the form of base calls. By contrast, Sanger sequencing uses the chain
termination method whereby 1% dideoxynucleotides (ddNTPs), nucleotides lacking a 3’-OH group required for DNA polymerization, are added with normal nucleotides to stop replication whenever a ddNTP is incorporated into the growing strand. Since this truncated replication repeats numerously, nucleotide polymers of varying lengths accumulate and are used to determine the position of each nucleotide in the sequence. The four ddNTPs are labeled with different fluorescent dyes that emit light at different wavelengths, which is captured and recorded on a chromatogram.

1.4 Research Objectives

One of the goals of this project includes demonstrating a difference in the microbial community profiles of the APC gut between individuals hatched in captivity and those hatched in the wild. Studies have shown that a normal colonization of bacteria occurs during the birth process and neonates are continuously exposed to microbes via food [38] and their surrounding environment. In domestic chickens, microbial colonization of the gastrointestinal tract begins immediately after hatch, and within 24 hours the small intestine has developed a bacterial population [39]. Hatching conditions vary considerably between captive and wild-hatched APCs and the young individuals are exposed to different bacteria. For example, the eggs are typically removed from the dam and placed into an incubator, where upon hatching the chicks are placed with a broody hen, while the wild-hatched chicks are in the presence of their birth hen throughout the process. Furthermore, the two release sites, Attwater’s Prairie Chicken National Wildlife Refuge (APCNWR) and Goliad, are very different environments and therefore it is expected that the microbial community profiles of the APC gut will change after release.
into the wild from captivity. APCNWR is a federally protected land while Goliad is private property and thus greater conservation management access to individuals is available at APCNWR. Studies have indicated the importance of early environmental exposure as a driver of microbial composition in the gut and that a shared environment leads to similar gut microbiota of cohabitating individuals [40].

Another objective of this project is to determine if microbial community profiles in the gut of captive APCs who have undergone antibiotic therapy are different from the baseline of the untreated captive APC, and if an increase in *Clostridia* is seen in the “treated” individuals. Antibiotic therapy has been shown to decrease the alpha diversity in the gut [41] and some antibiotics specifically predispose the gut to *C. difficile* infection. Antibiotic treatment causes the individual to be susceptible to subsequent *C. difficile* infection due to a lack of microbial diversity in the treated gut [42].

Finally, with respect to possible disruption in the cloacal microbiota with antibiotic therapy, recommendations for maintaining a healthy microbiota were described. The intestinal microbiota have an effect on both the innate and adaptive immune systems of the host [5] as well as in prevention of colonization by pathogens. The administration of antibiotics and other therapeutic agents at a young age may compromise the normal colonization of microflora and therefore adversely affect the development of the immune system. This slow or hindered development of the immune system may have prolonged effects on the “treated” individual, such as an inability or impaired ability to ward off infection after release into the wild. In addition, certain species of normal microflora can inhibit members of enteric bacteria, such as *Escherichia coli* and *Salmonella* sp. [43], members of which cause enterocolitis. The most
notorious antibiotic-associated pathogen responsible for a severe form of colitis and diarrhea in humans, *C. difficile*, is also inhibited by the normal gut microflora, but once this opportunistic pathogen is established it is very difficult to treat [23, 43, 44]. Probiotics have been shown to promote intestinal homeostasis between the gut microbiota and host [45], and are therefore one plausible recommendation as a supplement to the APC diet. Results from this work may provide valuable information and subsequent recommendations to the management program of these endangered birds. In addition, findings from this project may be applicable to other conservation management programs.
2.1 Sample Collection and Inclusion Guidelines

All Attwater’s prairie chicken (APC) individuals sampled pre-release were hatched in Spring 2011 and 2012 under captive breeding conditions at the Fossil Rim Wildlife Center (FRWC), the Houston Zoo, or the Abilene Zoo. All individuals selected for the antibiotics-treated studies were reared at FRWC. On the date sampling occurred, birds were crated to be transported to their release location. Individual birds were handled by trained APC conservation management staff and most samples were obtained by a veterinarian.

For the antibiotics studies, individual birds were chosen based on age and number of days post sulfadimethoxine water treatment (Table 2.1). Control and experimental groups were kept similar by taking the mean age of individuals and mean days post sulfadimethoxine-treated water administration of the samples in each group. All individuals, regardless of treatment, are administered sulfadimethoxine in the water for intestinal parasites such as coccidia, and are dewormed with fenbendazole and ivermectin. For the treated individuals, antibiotic therapy occurred within 3-9 days post-hatch, and for a minimum of 1-3 days of therapy. Birds were administered the drug enrofloxacin subcutaneously twice a day.

In the wild at Attwater’s Prairie-chicken National Wildlife Refuge and Goliad, the birds were randomly sampled by U.S. Fish and Wildlife staff using a night lighting technique [46]. The four APC individuals hatched in the wild were sampled at either the APCNWR or Goliad. The parents of the wild-hatched chicks were captive-reared, released, and mated randomly on their own (versus breeding pairs selected by the captive-breeding program).
The birds were handled ethically and appropriately to minimize stress and overheating. Here, the composition of the gut microbiota in APCs is being extrapolated through the use of cloacal sampling since direct sampling of the gut would endanger the animals. Each sample was collected using a Puritan™ sterile cotton-tipped applicator with plastic shaft and inserting the cotton tip into the cloaca the length of the cotton tip. Swabs were mixed with 500μl of tris-EDTA buffer in a 1.5ml sterile microcentrifuge tube and stored on dry ice in the field and during transport, and then transferred to the -80°C freezer upon return to the laboratory.

Table 2.1 Drug treatment history of birds used in the pooled study. Dark rows = pooled untreated (n = 7). White rows = pooled treated with Baytril (enrofloxacin) antibiotic (n = 5). Values indicate age of birds in days at time of intervention. Note all birds received sulfadimethoxine water treatment for intestinal parasites, and were dewormed with fenbendazole and ivermectin. NA, not applicable.

<table>
<thead>
<tr>
<th>Sample#</th>
<th>Gender</th>
<th>Enrofloxacin administered in days post-hatch</th>
<th>Sulfadimethoxine administered in days post-hatch</th>
<th>Sent to release in days post-hatch</th>
</tr>
</thead>
<tbody>
<tr>
<td>414</td>
<td>Male</td>
<td>NA</td>
<td>49-53</td>
<td>101</td>
</tr>
<tr>
<td>415</td>
<td>Male</td>
<td>NA</td>
<td>42-46</td>
<td>94</td>
</tr>
<tr>
<td>416</td>
<td>Male</td>
<td>NA</td>
<td>37-41</td>
<td>89</td>
</tr>
<tr>
<td>428</td>
<td>Male</td>
<td>NA</td>
<td>50-54</td>
<td>95</td>
</tr>
<tr>
<td>431</td>
<td>Male</td>
<td>NA</td>
<td>50-54</td>
<td>95</td>
</tr>
<tr>
<td>434</td>
<td>Male</td>
<td>NA</td>
<td>42-46</td>
<td>101</td>
</tr>
<tr>
<td>437</td>
<td>Male</td>
<td>NA</td>
<td>34-38</td>
<td>113</td>
</tr>
<tr>
<td>426</td>
<td>Male</td>
<td>9</td>
<td>50-54</td>
<td>109</td>
</tr>
<tr>
<td>427</td>
<td>Male</td>
<td>3-6</td>
<td>34-38</td>
<td>113</td>
</tr>
<tr>
<td>433</td>
<td>Male</td>
<td>4-6</td>
<td>46-50</td>
<td>105</td>
</tr>
<tr>
<td>436</td>
<td>Male</td>
<td>2, 4-6</td>
<td>40-44</td>
<td>119</td>
</tr>
<tr>
<td>446</td>
<td>Male</td>
<td>4-5</td>
<td>42-46</td>
<td>101</td>
</tr>
</tbody>
</table>

2.2 DNA Extraction

DNA was extracted from the cloacal samples using the FastDNA™ SPIN Kit for Soil by MP Biomedicals. Samples were thawed slowly on ice, vortexed briefly, and then centrifuged at
3,000xg for 3 minutes. The protocol suggests allowing for headspace in the lysing matrix tubes, therefore about 300μl of the supernatant was discarded. Sodium phosphate buffer was added to the sample tubes in order to maximize recovery of the sample and the pellet was resuspended by pipetting gently. The entire volume was transferred to the lysing matrix tube and each sample was homogenized using the FastPrep®-24 Instrument by MP Biomedicals. Two ethanol washes were carried out instead of one, and DNA was eluted in 100μl of nuclease-free molecular water after a five-minute incubation at 55°C. DNA was quantified using the NanoDrop-1000 spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA) to check for presence and purity of DNA.

2.3 Individually-Barcoded Wild Chick Analysis via Nested PCR

Four wild chicks were sampled by Susan Hammerly during a visit to Goliad and the APCNWR to collect cloacal samples on post-release individuals. Each sample was barcoded and pooled and then sequenced using one Ion 314 chip.

2.4 Full-length 16S rRNA Gene Amplification

Each 50μl PCR reaction was prepared with 20ng of template DNA, 1.0 U Phusion® High Fidelity DNA Polymerase (New England BioLabs®), 10μl of 5X Phusion® HF reaction buffer, 200μM of dNTPs, 3% (vol:vol) DMSO, and 0.5μM of each primer 27F and 1492R (Table 2.2). The cycling conditions used were: 98°C for 5 minutes, followed by 30 cycles of 98°C for 15 seconds, 56°C for 30 seconds, and 72°C for 1 minute, with a final extension time of 5 minutes at 72°C. PCR products were examined after electrophoresis on a 1% agarose gel at 80V for 30 minutes,
and the remaining PCR products were purified with the QIAquick® PCR Purification Kit by Qiagen, and quantified on the NanoDrop1000 to check for presence and purity of DNA.

2.4.1 V5 16S rRNA Gene Amplification

Each 50µl PCR reaction was prepared exactly as before, except 20ng of the initial PCR product was used as the DNA template, and 0.5µM of each barcoded forward primers IonAs1-E786F through IonAs4-E786F and IonP1-E989R (Table 2.2). The cycling conditions used were: 98°C for 3 minutes, followed by 25 cycles of 98°C for 15 seconds, and 61°C for 15 seconds, with a final extension time of 5 minutes at 72°C. PCR products were again examined after electrophoresis on a 1% agarose gel. This time, remaining PCR products were purified using Agencourt® AMPure® XP (Beckman Coulter) magnetic beads.

2.4.2 Library Preparation and Sequencing

The quality and quantity of DNA libraries were analyzed using the Experion™ Automated Electrophoresis Station (Bio-Rad Laboratories). Quality criteria used were absence of primer dimer or spurious bands and that the target band comprised at least 90% of the total concentration. Libraries were diluted to 26pM and emulsion PCR was carried out using the Ion OneTouch™ instrument and the Ion OneTouch™ ES, or enrichment system, powered by Ion Torrent™ according to the manufacturer’s instructions. Sequencing of the libraries was completed using a 314 chip on the Ion PGM™ system (Ion Torrent™, Life Technologies) according to the manufacturer’s instructions. After sequencing, reads were filtered to remove low quality and polyclonal sequences using default settings in Ion Reporter software version
4.1. Sequences containing the Ion Torrent 3’ adaptor were automatically trimmed. All quality control reviewed sequences were exported as standard flowgram format (SFF) files [36].

2.4.3 Sequence Analysis

SFF files were converted to FASTA and quality files using Galaxy [47]. The resulting FASTA and quality files were then inserted into RDP Pyrosequencing Pipeline using Pipeline Initial Process (http://pyro.cme.msu.edu/) [48]. This program removed the forward and reverse primers from each of the sequence fragments, any sequences under the 100bp threshold and any sequences with ambiguous nucleotides. This quality control ensured that only sequences that had the correctly sequenced primer, both forward and reverse, were included in the downstream parameters. The processed FASTA files were entered into RDP Classifier and placed at an 80% confidence threshold.

2.5 Enrofloxacin-Treated and Untreated Pooled Analysis of Captive-Bred APCs via Nested PCR

DNA samples were pooled in equimolar amounts into two respective groups based on antibiotic treatment or no treatment. Procedures were carried out same as described in 2.3.1, 2.3.2, 2.3.3, and 2.3.4 with the exception that the 2nd PCR was performed using IonA-E786F and IonP1-E989R (Table 2.1) with no barcodes. Each pooled group was treated separately and sequenced using Ion 314 chips. Since the samples were pooled into two groups, the means could not be compared, as there was only one datum per group.

Aligned sequences from each group were generated with their individual cluster files based on the RDP pyrosequencing pipeline. The cluster files produced were used to generate
rarefaction curves that defined the number of OTUs defined at 97% similarity level with respect to total number of reads for each sample.

2.5.1 Preparation of Clone Libraries for Sanger Sequencing

The individual DNA samples selected for the treatment and no treatment groups were again pooled and 20ng was amplified as before using primers 27F and 1492R with the illustra™ puReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) in a 25μl reaction. Thermal cycling conditions used were: 95°C for 5 minutes, followed by 30 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 1.5 minutes, with a final extension time of 20 minutes at 72°C. PCR products were examined for quality on a 1% agarose gel and then cleaned and quantified as before. PCR products were then cloned into the PCR® 2.1-TOPO® DNA vector following the TOPO® TA Cloning® protocol (Life Technologies). The cloned vectors for each group were transformed into chemically competent OneShot® TOP10 E. coli cells (Life Technologies). After successful blue/white screening, twenty isolated and genetically modified E. coli colonies containing the gene insert were randomly selected and plasmids were prepared from subcultures using the FastPlasmid™ Mini kit (5 Prime). Plasmids were sequenced at UNT in Dr. Jeff Johnson’s Lab via the chain termination method using primer M13R (Table 2.2) on a 3130xl ABI Sequencer with BigDye Terminator chemistry (Applied Biosystems®). FASTA sequences were analyzed using NCBI BLAST [49] nucleotide collection and compared to output on RDP Classifier [50].
2.6 Comparison of Individuals Pre- and Post-Release

Individuals were randomly sampled at the APCNWR approximately six months after being transported from the captive-breeding facility and released. Three individuals sampled during this trip had previously been sampled pre-release and therefore a pre- and post-release analysis was available. Procedures were carried out as described in 2.3.1, 2.3.2, 2.3.3, and 2.3.4 with barcoded forward primers As1IonA-E786F through As4IonA-E786F (Table 2.2) in the nested PCR. The last barcode was used for a pooled sample of the three pre- or post-release individuals. The four barcoded libraries for each analysis, pre- or post-release, were pooled and sequenced on two Ion 314 chips.

2.7 Analyses of Individual Samples of Untreated and Treated Captive-Bred APCs

Two separate sequencing experiments occurred for the treatment/untreated analysis in attempt to determine if there is a difference in microbial composition of the APC cloaca of captive individuals. With the first set of samples, DNA template was eventually exhausted with the various analyses, repeated sequencing due to lack of coverage, and through troubleshooting. Therefore, a second group of individuals was selected based on the same criteria as before (Table 3.1). For both analyses, nested PCR was not performed but rather a single PCR amplification of the V4 region of the 16S gene.

With the availability of a greater number of barcodes, all samples were investigated individually on a single sequencing chip. The first sequencing analysis included the same 12 samples that were pooled into the two groups, treated and untreated, in the above-described
analysis (2.4) and sequenced on an Ion 314 chip. The second analysis included 10 newly selected individuals (Table 3.1) from a repository of previously stored samples of APCs.

2.7.1 V4 16S rRNA Gene Amplification and Library Preparation

A single PCR reaction was performed for each sample instead of nested PCR as performed in the previous analyses. Each sample was prepared in triplicate, 25µl reactions, using 20ng of original DNA template, 0.5U Phusion® High Fidelity DNA Polymerase (New England BioLabs®), 5µl of 5X Phusion® HF reaction buffer, 200µM of dNTPs, and 3% (vol:vol) DMSO. For the first analysis, 0.5µM of each barcoded forward primers 1IonA-515F through 12IonA-515F and IonP1-806R (Table 2.2) reverse primer were used, and for the second analysis, 0.5µM of each barcoded forward primers 1IonA-515F through 10IonA-515F and IonP1-806R (Table 2.2) reverse primer were used. The cycling conditions used were: 98°C for 3 minutes, followed by 30 cycles of 98°C for 10 seconds, and 52°C for 15 seconds, 72°C for 15 seconds, with a final extension time of 5 minutes at 72°C. PCR products were examined following electrophoresis on a 1% agarose gel and then the remaining volumes for each replicate were pooled into one tube for each sample. For the first analysis, the samples were purified with the QIAquick® PCR Purification Kit by Qiagen, and for the second analysis the samples were purified using Agencourt® AMPure® XP (Beckman Coulter) magnetic beads. All samples were quantified on the NanoDrop1000 to check for presence and purity of DNA.

2.7.2 Library Preparation and Sequencing

Quality and quantity of DNA libraries were analyzed using the Bioanalyzer®
electrophoresis assay (Agilent© Technologies) for the first analysis, or either the Bioanalyzer® or the Experion™ for the second analysis under the same criteria as described in 2.3.3. Any samples that still exhibited spurious PCR products were gel purified using the Zymoclean™ Gel DNA Recovery Kit by Zymo Research, and this was utilized for six out of 12 samples in the first analysis but was not necessary for the second analysis. Any gel-purified samples were once again quantified on the Bioanalyzer®.

Once purified, libraries were diluted to 26pM, pooled into one reaction chamber, and emulsion PCR was carried out using the Ion OneTouch2™ instrument. The ion sphere particles were assessed for template quality using the Ion Sphere™ Quality Control Kit and Qubit® 2.0 fluorometer (Life Technologies). The templated spheres were enriched on the Ion OneTouch™ ES, or enrichment system, powered by Ion Torrent™ according to the manufacturer’s instructions. Sequencing of the libraries was completed using a 314 chip (first analysis) and 316 chip (second analysis) on the Ion PGM™ system powered by Ion Torrent™ according to the manufacturer’s instructions. After sequencing, the reads were filtered to remove low quality and polyclonal sequences by proprietary software. Any sequences containing the Ion Torrent 3’ adaptor were automatically trimmed. All quality control reviewed sequences were exported as standard flowgram format or SFF files [36].

2.7.3 Sequence and Statistical Analysis

SFF files from barcoded individual samples were converted to FASTA and quality files, and subsequent analysis was carried out using the Mothur pipeline which takes in barcoded sequence reads and separates them into individual communities by barcode [51]. Sequences
were aligned and classified using the RDP Classifier, clustered and distance measurements were calculated all within the Mothur pipeline. Once sequences were grouped into OTUs at 97% similarity, diversity estimation indices (Shannon, Chao1, ACE, and Evenness) were calculated in Mothur. Branch lengths within a phylogenetic tree were determined using UniFrac [52] and principal coordinates analysis (PCoA) was used to identify any specific factors that drive differences among communities.

Table 2.2 List of primers used for PCR amplification. No change in font format is the portion of the sequence homologous to the gene of interest. For the NGS primers, the bold portion includes Ion Torrent specific adaptor sequence and the underlined portion includes the identifying barcode.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Description</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F</td>
<td>Forward primer universal region full-length 16S rRNA gene</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
</tr>
<tr>
<td>1492R</td>
<td>Reverse primer universal region full-length 16S rRNA gene</td>
<td>ACCTTGTTACGACTT</td>
</tr>
<tr>
<td>IonA-E786F</td>
<td>Forward primer V5 region 16S rRNA gene w/ Ion Torrent specific adaptor</td>
<td>CCATCTCATCCCTGCGTGTCTCCGACTCAGGATTAGATACCTGGTAG</td>
</tr>
<tr>
<td>IonP1-E989R</td>
<td>Reverse primer V5 region 16S rRNA gene w/ Ion Torrent specific adaptor</td>
<td>CCTCTCTATGGGCGAGTCGGTGATCTTGTCGGGGCCCCGGTCAATTCC</td>
</tr>
<tr>
<td>M13R</td>
<td>Universal primer for cloning vectors</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>IonAs1-E786F</td>
<td>Forward primer V5 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCCCTGCGTGTCTCCGACTCAGCTGATTAGATACCTGGTAG</td>
</tr>
<tr>
<td>IonAs2-E786F</td>
<td>Forward primer V5 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCCCTGCGTGTCTCCGACTCAGTAGATTAGATACCTGGTAG</td>
</tr>
<tr>
<td>IonAs3-E786F</td>
<td>Forward primer V5 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCCCTGCGTGTCTCCGACTCAGAAAGATTAGATACCTGGTAG</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Description</td>
<td>Primer Sequence</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>IonAs4-E786F</td>
<td>Forward primer V5 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCTCGTGTTCTCCGACTCAGCA GATTAGATACCTGGTAA</td>
</tr>
<tr>
<td>1ionA-515F</td>
<td>Forward primer V4 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCTCGTGTTCTCCGACTCAG CTAAGGTAAACGTGGCCAGCMGGCGCGGTAA</td>
</tr>
<tr>
<td>2ionA-515F</td>
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</tr>
<tr>
<td>3ionA-515F</td>
<td>Forward primer V4 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCTCGTGTTCTCCGACTCAG AAGAGGATACCTGGCAGCMGGCGCGGTAA</td>
</tr>
<tr>
<td>4ionA-515F</td>
<td>Forward primer V4 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
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<tr>
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<td>6ionA-515F</td>
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<td>7ionA-515F</td>
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<td>CCATCTCATCCCTCGTGTTCTCCGACTCAG TTCGTGATTACCTGGCAGCMGGCGCGGTAA</td>
</tr>
<tr>
<td>8ionA-515F</td>
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<td>10ionA-515F</td>
<td>Forward primer V4 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
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<tr>
<td>11ionA-515F</td>
<td>Forward primer V4 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCTCGTGTTCTCCGACTCAG CTGACCGGAAACGTGGCAGCMGGCGCGGTAA</td>
</tr>
<tr>
<td>12ionA-515F</td>
<td>Forward primer V4 region 16S rRNA gene w/ Ion Torrent specific adaptor and barcode</td>
<td>CCATCTCATCCCTCGTGTTCTCCGACTCAG TAGGATGATTACCTGGCAGCMGGCGCGGTAA</td>
</tr>
</tbody>
</table>
3.1 Fluoroquinolone-Treated and Untreated Pooled Analysis of Captive-Bred Attwater’s Prairie Chickens (APCs)

The samples used in this study were chosen based on similar age at the time of release, similar length of time after receiving sulfadimethoxine water to time of release, and if treated then the enrofloxacin was administered within the first 10 days after hatch and therapy typically lasted approximately 1-3 days. The “Untreated” pooled group included 7 individuals denoted: 414, 415, 416, 428, 431, 434, and 437. The “Treated” pooled group included 5 individuals denoted: 426, 427, 433, 436, and 446. This pooled analysis occurred prior to the availability of barcoding capability for the Ion Torrent™.

Semiconductor sequencing yielded 79,823 sequence reads across the two groups of pooled samples after preprocessing and quality checks. The predominant phyla for both groups were Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Figure 3.1). While there was a minimal decrease in Firmicutes in the treated group, there was a 43% decrease in Bacteroidetes in the treated group from the untreated control group. Conversely, the treated group increased by 28% in Proteobacteria, a group that includes many of the colitis-causing enteric species.

The greatest membership for both groups is comprised of the classes Actinobacteria, Alphaproteobacteria, Bacilli, Clostridia, Erysipelotrichia, Gammaproteobacteria, and unclassified Firmicutes (Figure 3.2). At the class level, the treated group had more diversity than the untreated group, which was dominated 80% by Bacilli. Compared to the untreated
control group, the treated group showed a decrease in Bacilli by 41%, an increase in Clostridia by 86%, an increase Erysipelotrichia by 55%, and an increase of 32% in Gammaproteobacteria.

![Figure 3.1](image)

*Figure 3.1*. Microbial community profiles of the cloaca of the untreated and treated groups of pooled APC samples at the phylum taxonomic level. “Other” included phyla Acidobacteria, Nitrospira, Spirochaetes, and Verrucomicrobia each comprising less than 1% cumulative abundance in each sample. Note the y-axis scale starts at 80% and the remaining below 80% is all Firmicutes.

The primary orders for both groups that comprised the classes at greater than 1% cumulative abundance include: Alteromonadales, Bacillales, Clostridiales, Enterobacterales, Erysipelotrichiales, Lactobacillales, Rhizobiales, and unclassified Bacilli (Figure 3.3). The untreated control group was dominated by Bacillales and Lactobacillales. On the contrary, the treated group was dominated by Clostridiales and Erysipelotrichiales. The treated group decreased in Bacillales by 48.5% and Lactobacillales by 30%, but increased by 87% each in Enterobacterales and Clostridiales.
Figure 3.2. Microbial community profiles of the cloaca of the untreated and treated groups of pooled APC samples at the class taxonomic level, further subdivided by the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. “Other” included classes Betaproteobacteria, Bacteroidia, Deltaproteobacteria, Flavobacteria, Negativicutes, Sphingobacteria, unclassified Bacteroidetes, and unclassified Proteobacteria each comprising less than 1% cumulative abundance in each group. Classes of the phyla Acidobacteria, Nitrospira, Spirochaetes, and Verrumicrobia were not included in this class analysis since these phyla were less than 1% abundance in the phylum analysis.

Figure 3.3. Microbial community profiles of the cloaca of the untreated and treated groups of pooled APC samples at the order taxonomic level, further subdivided by the classes Bacilli, Erysipelotrichia, Gammaproteobacteria, Clostridia, Actinobacteria, and Alphaproteobacteria. Other included the orders Acidimicrobiales, Actinomycetales, Aeromonadales,
Alphaproteobacteria_incertae_sedis, Bifidobacteriales, Coriobacteriales, Oceanospirillales, Pseudomonadales, Rhodobacterales, Rhodospirillales, Solirubrobacterales, Sphingomonadales, Xanthomonadales, and unclassified Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Clostridia. Orders of the classes Betaproteobacteria, Bacteroidia, Deltaproteobacteria, Flavobacteria, Negativicutes, Sphingobacteria, and unclassified Bacteroidetes and Proteobacteria were not included in this order analysis since they were less than 1% abundance in the class analysis.

3.1.1 Microbial Richness between the Treated and Untreated Groups

Microbial richness was defined based on the number of operational taxonomic units, or OTUs, identified for each group of pooled samples. Sequences from each group were aligned and individual cluster files were generated based on the RDP pyrosequencing pipeline. The cluster files produced were used to generate rarefaction curves that defined the number of OTUs at 97% similarity level with respect to total number of reads for each group (Figure 3.4). Microbial richness was significantly greater in the treated group ($p < 0.01$, ANCOVA).

![Figure 3.4. Rarefaction curves for treated (red) and untreated (blue) groups of pooled samples at 97% similarity between operational taxonomic units.](image)
3.1.2 Microbial Diversity of Clones for Treated and Untreated Groups of Pooled Samples

Twenty clones with near-full-length 16S rDNA for each group were randomly selected, then prepared and sequenced. Sanger sequencing of each clone yielded 16 sequences for the untreated group and 20 sequences for the treated group. Sequences were analyzed using BLAST and RDP Classifier. The sequence matches from RDP Classifier confirmed a shift from Bacillales in the untreated control group to Clostridiales in the treated group (Figure 3.5 and Figure 3.6).

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**Figure 3.5.** Hierarchical view from RDP Classifier at 80% confidence for all 16 sequences in the untreated group clones.

The clone library for the untreated control group comprised 50% Bacillales with no representation from Enterobacterales, while the treated group comprised 50% Clostridiales and 5% Enterobacterales (Figure 3.7 and Figure 3.8). The full-length sequence of the closest match was used to generate maximum likelihood phylogenetic trees for each group (Figure 3.9 and Figure 3.10). Eight of the nineteen successful sequences for the treated group clustered
within the genus *Clostridium* and were identified as *C. bartlettii* and *C. glycolicum*. Most notable was the single occurrence of *Shigella flexneri*, a causative enteric of diarrhea and dysentery in humans, in the treated clone library.

**Figure 3.6.** Hierarchical view from RDP Classifier at 80% confidence for all 20 sequences in the treated group clones.

**Figure 3.7.** Relative abundances of phylotypes at 80% confidence at the order taxonomic level for the untreated group of 16 clones.
Figure 3.8. Relative abundances of phylotypes at 80% confidence at the order taxonomic level for the treated group of 20 clones.

Figure 3.9. Phylogenetic tree of the closest BLAST matches to near-full-length 16S sequences for 16 clones in the untreated control group. Sequences were aligned using MEGA5 and inserted into a bootstrapped phylogenetic tree using maximum likelihood. Sequences without the “clone” designation represent NCBI sequences added as reference species.
3.10 Phylogenetic tree of the closest BLAST matches to near-full-length 16S sequences for 19 clones in the treated group. Sequences were aligned using MEGA5 and inserted into a bootstrapped phylogenetic tree using maximum likelihood. Sequences without the “clone” designation represent NCBI sequences added as reference species.

3.2 Analyses of Individual Samples of Untreated and Treated Captive-Bred APCs

The first analysis of twelve samples discussed in the beginning of this section included the untreated individuals 414, 415, 416, 428, 431, 434, and 437 and the treated individuals 426, 427, 433, 436, and 446, all of which were previously examined in the pooled treated/untreated analysis described in section 3.1. All individuals were hatched in 2011, reared at FRWC, and samples were acquired pre-release. As a result of numerous attempts at sequencing, whether in the pooled analysis or the individual analysis, the finite amount of template DNA was depleted. Therefore, after troubleshooting and optimization of protocols, a second group of individuals were selected based upon similar criteria as described in Table 2.1. The second
analysis of ten samples included the untreated individuals 520, 525, 541, 545, and 595 and the treated individuals 513, 532, 693, 699, and 704 (Table 3.1), none of which had been previously analyzed. All of these individuals were hatched in 2012, reared at FRWC, and samples were acquired pre-release.

3.2.1 First Analysis of Individual Samples of Treated and Untreated Captive-Bred APCs

Semiconductor sequencing yielded 57,628 sequence reads from the twelve barcodes for the first analysis of treated and untreated individuals. The predominant phylum by over 82% across all samples was Firmicutes (Figure 3.11). At this level there was no pattern observed between the samples of the two groups, treated and untreated. Samples 415-N and 436-Y have microbial community profiles most similar to each other, even though one individual received antibiotic treatment and the other did not. The evenness, as described in relative abundances, between the treated and untreated groups of samples was not found to be significantly different at the phylum level ($p = 0.9$, non-parametric ANOVA).

Bacilli was the predominant class in all but two individuals, 431-N and 446-Y, where Clostridia dominated in 431-N and both Clostridia and Erysipelotrichia were co-dominant in 446-Y (Figure 3.12). Once again, there was no pattern associated within each group of individual samples, except that two of the treated samples, 426-Y and 446-Y, had an increased abundance in Erysipelotrichia at 41% and 45% of cumulative abundance, respectively. The predominant orders were Bacillales, Lactobacillales, Clostridiales, and Erysipelotrichiales (Figure 3.13). Within the class of Bacilli, Lactobacillales and Bacillales were the most abundant and found in 8 out of the 12 samples, 414-N, 416-N, 428-N, 434-N, 437-N, 427-Y, 433-Y, and 436-Y.
Sample 431-N was more abundant in Clostridiales at 74% than any other order. Sample 415-N had near equal abundance in Lactobacillales (43%) and Clostridiales (32%). Sample 426-Y had equally high membership from Erysipelotrichiales and Bacillales at 41% and 40%, respectively. And sample 446-Y exhibited highest abundances of Erysipelotrichiales and Clostridiales at 45% and 44%, respectively.

Microbial richness was defined based on the number of OTUs at 97% sequence similarity identified in each of the individual samples. Sequences from each sample were aligned and clustered using a maximum distance of 3% and placed into rarefaction curves (Figure 3.14). While the treated individual, sample 426-Y, had the highest number of OTUs than the other samples, there was no significant difference in microbial richness between the treated and untreated individuals ($p = 0.8$, student’s t test). Furthermore, the Shannon diversity indices, which take both richness and evenness into account, were not significantly different between the two groups ($p = 0.9$, student’s t test).

Table 3.1 Individuals selected and described by sample number and organized by treatment and analysis. An “M” or “F” after each sample number indicated the gender, male or female, respectively.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Individuals untreated (Sample #)</th>
<th>Individuals treated (Sample #)</th>
<th>Year of Hatch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second</td>
<td>520 F 525 M 541 F 545 F 595 F</td>
<td>513 M 532 F 693 F 699 M 704 M</td>
<td>2012</td>
</tr>
</tbody>
</table>
Figure 3.11. Microbial community profiles of the cloaca of the untreated and treated individual APC samples analyzed at the phylum taxonomic level for the first group of samples 414, 415, 416, 426, 427, 428, 431, 433, 434, 436, 437, and 446. The “N” or “Y” after each sample number indicated the group untreated or treated, respectively. “Other” included phyla Acidobacteria, Armatimonadetes, Bacteroidetes, Chlamydiae, Chloroflexi, Cyanobacteria_Cloroplast, Deinococcus-Thermus, Gemmatimonadetes, Nitrospira, Planctomycetes, Verruromicrobia, unclassified Bacteria, and the candidate division phylum OD1 each comprising ≤2% cumulative abundance in each sample.

Figure 3.12. Microbial community profiles of the cloaca of the untreated and treated individual APC samples analyzed at the class taxonomic level for the first group of samples 414, 415, 416, 426, 427, 428, 431, 433, 434, 436, 437, and 446. The “N” or “Y” after each sample number
indicated the group untreated or treated, respectively. “Other” included classes Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, and Negativicutes, each comprising less than 2% cumulative abundance in each sample. Also, the classes for phyla that comprised ≤2% in the phylum analysis were not included in this class analysis.

Figure 3.13. Microbial community profiles of the cloaca of the untreated and treated individual APC samples analyzed at the order taxonomic level for the first group of samples 414, 415, 416, 426, 427, 428, 431, 433, 434, 436, 437, and 446. The “N” or “Y” after each sample number indicated the group untreated or treated, respectively. “Other” included orders Acidimicrobiales, Rubrobacterales, Solirubrobacterales, unclassified Actinobacteria, Alphaproteobacteria_order_incertae_sedis, Caulobacterales, Rhodobacterales, Rhodospirillales, Rickettsiales, Sphingomonadales, unclassified Alphaproteobacteria, Burkholderiales, Neisseriales, Rhodocyclales, unclassified Betaproteobacteria, Aeromonadales, Pasteurellales, Pseudomonadales, Xanthomonadales, unclassified gammaproteobacteria, unclassified Proteobacteria, unclassified Bacilli, and unclassified order of unclassified phylum, each comprising less than 1% cumulative abundance in each sample. Also, the orders for classes that comprised less than 2% in the class analysis were not included in this class analysis.
Figure 3.14. Rarefaction curves at 97% similarity between operational taxonomic units for untreated and treated individual APC samples for the first group of samples 414, 415, 416, 426, 427, 428, 431, 433, 434, 436, 437, and 446. The “N” or “Y” after each sample number indicated the group untreated or treated, respectively. Curves for untreated samples were dashed lines and curves for treated samples were solid lines.

3.2.2 Second Analysis of Individual Samples of Treated and Untreated Captive-Bred APCs

Semiconductor sequencing yielded 463,994 sequence reads between the ten barcodes for the second analysis of treated and untreated individuals. The phyla represented at greater than 1% were Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and unclassified phylum of Bacteria (Figure 3.15). The predominant phyla were Actinobacteria and Firmicutes at 34% and 60%, respectively, of the cumulative abundance across all ten samples. Sample 595-N stood out with a higher membership from Bacteroidetes at 7.5% while all other samples had less than 1%. With the exception of sample 532-Y, all of the treated individuals had a greater abundance of Firmicutes than did the untreated individuals. The evenness, as described in relative abundances, between the treated and untreated groups of samples was not found to be significantly different at the phylum level ($p = 0.3$, ANOVA).
The classes represented at greater than 1% were Actinobacteria, Bacteroidia, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacilli, Clostridia, Erysipelotrichia, Negativicutes, unclassified Bacteroidetes, unclassified Firmicutes, and unclassified Proteobacteria (Figure 3.16). The predominant classes for all samples were Actinobacteria, Bacilli, and Clostridia. With the exceptions of the untreated individual, 595-N, the treated individuals exhibited the highest membership from Clostridia. However, the treated individual, 704-Y, had less than 1% Clostridia. At the family level, the treated individuals demonstrated greater evenness than did the untreated individuals (Figure 3.17). The representatives of Clostridiales seen primarily in the treated samples plus untreated sample 595-N comprised the families of Lachnospiraceae and Ruminococcaceae. The predominant families for the untreated samples were Corynebacteriaceae, Planococcaceae, Staphylococcaceae, and Streptococcaceae, with the latter three members of Bacilli. Notably, *Corynebacterium* is present across all samples but is more prevalent in the untreated individuals.

Microbial richness was defined based on the number of OTUs at 97% sequence similarity identified in each of the individual samples. Sequences from each sample were aligned and clustered using a maximum distance of 3% and placed into rarefaction curves (Figure 3.18). While there three of the five treated samples were the most rich as seen in the rarefaction curves, the microbial richness between the treated and untreated groups was not significantly different ($p = 0.3$, student’s t test). Diversity estimates were calculated for each sample. The Shannon diversity indices exhibited a pattern of higher numbers for the treated samples, with the exceptions of 704-Y with the third lowest value and 595-N with the third
highest value (Table 3.2). Higher Shannon values indicate greater diversity. The diversity measured in Shannon values was not found to be significantly different between the treated and untreated groups in the second analysis ($p = 0.2$, student’s $t$ test).

Differences in cloacal microbial community composition between treated and untreated samples were assessed using a phylogeny-based metric, UniFrac. Sequences for each sample were clustered and weighted UniFrac distance measurements were calculated in Mothur. Individual samples for each group, treated and untreated, were expected to cluster together; however, that was not the case as shown in Figure 3.19 for the weighted UniFrac distances for each sample. The only two untreated samples most closely related were 545 and 525, and the only two treated samples most closely related were 513 and 693. In contrast, UniFrac-based principal coordinates analysis (PCoA) exhibited clustering of four of the five treated samples (Figure 3.20). The PCoA was used to identify specific environmental variables that drive differences among communities. It is a plot of the weighted UniFrac distance metrics on different axes and used as a visual representation of data.

The untreated individuals selected for the second analysis exhibited a greater mean length of days survived post-release at $319.2 \pm 6.3$ days to $146.8 \pm 98.0$ days for the treated individuals and this difference was found to be significant ($p < 0.05$, student’s $t$ test, heteroscedastic variance accounted for).
Figure 3.15. Microbial community profiles of the cloaca of the untreated and treated individual APC samples analyzed at the phylum taxonomic level for the second group of samples 513, 520, 525, 532, 541, 545, 595, 693, 699, and 704. The “N” or “Y” after each sample number indicated the group untreated or treated, respectively. “Other” included phyla Acidobacteria, Armatimonadetes, Chlamydiae, Chloroflexi, Deinococcus-Thermus, Elusimicrobia, Fusobacteria, Gemmatimonadetes, Lentisphaerae, Nitrospira, Planctomycetes, Synergistetes, Tenericutes, Verrumicrobia, and the candidate division phylum TM7 each comprising less than 1% relative abundance in each sample.

Figure 3.16. Microbial community profiles of the cloaca of the untreated and treated individual APC samples analyzed at the class taxonomic level for the second group of samples 513, 520, 525, 532, 541, 545, 595, 693, 699, and 704. The “N” or “Y” after each sample number indicated the group untreated or treated, respectively. “Other” included classes Acidobacteria_Gps2-4,
6, 10, and 16, Anaerolineae, Armamonadetes_Gp5, Bacteroidetes_incertae_sedis, Caldilineae, Chlamydiae, Deinococci, Deltaproteobacteria, Elusimicrobia, Epsilonproteobacteria, Flavobacteria, Fusobacteria, Gemmatimonadetes, Ktedonobacteria, Lentisphaeria, Mollicutes, Nitrospira, Opitutae, Planctomycetacia, Sphingobacteria, Synergistia, Tenericutes, Thermomicrobia, Verrumicrobiae, and TM7_incertae_sedis, each comprising less than 1% relative abundance in each sample.

**Figure 3.17.** Microbial community profiles of the cloaca of the untreated and treated individual APC samples analyzed at the family taxonomic level for the second group of samples 513, 520, 525, 532, 541, 545, 595, 693, 699, and 704. The “N” or “Y” after each sample number indicated the group untreated or treated, respectively. The families included in the “Other” group each comprised less than 1% relative abundance in each sample.
Figure 3.18. Rarefaction curves at 97% similarity between operational taxonomic units for untreated and treated individual APC samples for the second group of samples 513, 520, 525, 532, 541, 545, 595, 693, 699, and 704. The “N” or “Y” after each sample number indicated the group untreated or treated respectively. Curves for untreated samples were dashed lines and curves for treated samples were solid lines.

Figure 3.19. Hierarchical clustering of untreated and treated individual APC samples for the second group of samples. The bar represents a weighted UniFrac distance of 0.02. The “N” or “Y” after each sample number indicated the group untreated or treated respectively.
Figure 3.20. Principal coordinate analysis of the weighted UniFrac distance measurements for untreated and treated individual APC samples for the second group of samples. The untreated samples were represented with blue circles while the treated samples were represented with red circles. The “N” or “Y” after each sample number indicated the group untreated or treated, respectively.

Table 3.2 Diversity indices for untreated and treated individual APC samples for the second group of samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No of sequences</th>
<th>No of OTUs (97%)</th>
<th>Coverage</th>
<th>Shannon</th>
<th>ACE</th>
<th>Chao1</th>
<th>Evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>520-N</td>
<td>34397</td>
<td>782</td>
<td>98.2%</td>
<td>1.47</td>
<td>7850</td>
<td>3813</td>
<td>0.221</td>
</tr>
<tr>
<td>525-N</td>
<td>34397</td>
<td>1175</td>
<td>97.2%</td>
<td>1.65</td>
<td>12368</td>
<td>5361</td>
<td>0.233</td>
</tr>
<tr>
<td>541-N</td>
<td>34397</td>
<td>1525</td>
<td>96.5%</td>
<td>2.12</td>
<td>16336</td>
<td>6468</td>
<td>0.290</td>
</tr>
<tr>
<td>545-N</td>
<td>34397</td>
<td>1553</td>
<td>96.4%</td>
<td>1.93</td>
<td>14857</td>
<td>6796</td>
<td>0.263</td>
</tr>
<tr>
<td>595-N</td>
<td>34397</td>
<td>3719</td>
<td>91.2%</td>
<td>3.98</td>
<td>49851</td>
<td>19169</td>
<td>0.484</td>
</tr>
<tr>
<td>513-Y</td>
<td>34397</td>
<td>1489</td>
<td>96.7%</td>
<td>2.16</td>
<td>14098</td>
<td>5219</td>
<td>0.296</td>
</tr>
<tr>
<td>532-Y</td>
<td>34397</td>
<td>2737</td>
<td>93.6%</td>
<td>3.52</td>
<td>29943</td>
<td>12321</td>
<td>0.444</td>
</tr>
<tr>
<td>693-Y</td>
<td>34397</td>
<td>3715</td>
<td>91.6%</td>
<td>4.48</td>
<td>36194</td>
<td>16149</td>
<td>0.545</td>
</tr>
<tr>
<td>699-Y</td>
<td>34397</td>
<td>3522</td>
<td>91.9%</td>
<td>4.61</td>
<td>32751</td>
<td>14775</td>
<td>0.564</td>
</tr>
<tr>
<td>704-Y</td>
<td>34397</td>
<td>1291</td>
<td>96.9%</td>
<td>1.90</td>
<td>15101</td>
<td>5985</td>
<td>0.265</td>
</tr>
</tbody>
</table>
3.3 Individually-Barcoded Wild Chick Analysis

The four wild chicks were approximately six weeks of age at the time of sampling. While the chicks were hatched in the wild, the parents were all captive-reared. The chicks had not previously undergone any antibiotic therapy at the time of sampling and therefore the results shown here are based on the environmental conditions at the time of hatching. One of the chicks sampled hatched at APCNWR and the other three chicks sampled hatched at Goliad.

Semiconductor sequencing yielded 79,965 sequence reads across the four individually-barcoded samples after preprocessing and quality checks. Common to all four chicks was a preponderance of the phylum Proteobacteria with greater than 80% of the total abundance, followed by Firmicutes. The predominant orders in this data set were: Actinomycetales, Alteromonadales, Bacillales, Burkholderiales, Clostridiales, Enterobacteriales, Lactobacillales, and Oceanospirillales.

Only one sample from APCNWR, As1.500.APCNWR, had less than 1% abundance of Enterobacteriales while the three from Goliad ranged from 7.5-50% abundance. In addition, the presence of Burkholderiales decreased by nearly half for the three Goliad chicks compared to the APCNWR chick. All members of the class Bacilli for sample As4.512.Goliad were Lactobacillales, which was different from the other 3 samples that had both Bacillales and Lactobacillales.
Figure 3.21. Microbial community profiles of the cloaca of four wild-hatched APC chicks approximately six weeks old at the phylum taxonomic level. “Other” included phyla Acidobacteria, Bacteroidetes, and unclassified Bacteria each comprising less than 1% cumulative abundance in each sample. The first wild chick hatched at APCNWR while the last three wild chicks hatched at Goliad.

Figure 3.22. Microbial community profiles of the cloaca of four wild-hatched APC chicks approximately six weeks old at the order taxonomic level. “Other” included orders Bacteroidales, Coriobacterales, Legionellales, Neisseriales, Pseudomonadales, Rhizobiales,
Rhodocyclales, Rhodospirillales, Selenomonadales, Solirubrobacterales, Sphingobacterales, Sphingomonadales, Vibrionales, unc Acidobacteria_Gp1, unc Actinobacteria, unc Alphaproteobacteria, unc Bacilli, unc Betaproteobacteria, unc Clostridia, and unc Gammaproteobacteria, each comprising less than 1% cumulative abundance in each sample. The first wild chick hatched at APCNWR while the last three wild chicks hatched at Goliad.

3.4 Comparison of Individuals Pre- and Post-Release

Not all of the individuals were re-sampled in the wild post-release due to mortality and random sampling. The individuals re-sampled post-release were 451, 455, and 486 and correspond to 414, 415, and 434 respectively. Henceforth, these will be denoted 414/451, 415/455, and 434/486 when discussing the individual bird. All three individuals were previously investigated under the untreated control group. The individuals 414/451 and 415/455 were released to APCNWR while individual 434/486 was released to Goliad. All three birds survived 181, 181, and 174 days post-release, respectively.

Semiconductor sequencing yielded 11, 500 sequence reads between the four barcodes for the “Pre-release” group and 179,500 sequences between the four barcodes for the “Post-release” group. Firmicutes and Proteobacteria were the predominant phyla for all samples (Figure 3.23). The other phyla that comprised greater than 1% represented in all eight samples included Actinobacteria, Bacteroidetes, and unclassified Bacteria. Two of the samples, Pre.415/455.APCNWR and Pre.434/486.Goliad, had a preponderance of Proteobacteria over Firmicutes. Notably, these birds also had higher membership of Bacteroidetes pre-release, and in both samples the membership from Bacteroidetes all but disappeared post-release. Interestingly, these same two birds had a similar profile pre-release, but the profiles were very different post-release, which may be attributed to environmental differences between the
release sites of APCNWR and Goliad. The two individuals released to APCNWR 414/451 and 415/455 increased in abundance of Firmicutes post-release while the individual released to Goliad 434/486 increased in abundance of Proteobacteria.

The classes represented at greater than 2% cumulative abundance were Actinobacteria, Alphaproteobacteria, Bacilli, Betaproteobacteria, Clostridia, Flavobacteria, Gammaproteobacteria, unclassified Firmicutes, and unclassified Proteobacteria (Figure 3.24). The two individuals released to APCNWR 414/451 and 415/455 increased in abundance of Bacilli post-release while the abundance of Gammaproteobacteria decreased post-release. In addition, while these two individuals had dissimilar microbial community profiles pre-release, their profiles equalized in the same environment post-release. As expected, the individual released to Goliad 434/486 had a different profile post-release even though it shared a similar pre-release profile to the individual 415/455.

The predominant orders represented for both pre- and post-release samples were Alteromonadales, Bacillales, Burkholderiales, Chromatiales, Clostridiales, Enterobacteriales, Lactobacillales, Oceanospirillales, unclassified Bacilli, and unclassified Betaproteobacteria (Figure 3.25). At the order level, none of the individuals had similar profiles pre-release. The pre-release sample Pre.414/451.APCNWR was dominated with Lactobacillales, Oceanospirillales, and Bacillales, while Pre.415/455.APCNWR was dominated with Burkholderiales and Clostridiales, and Pre.434/486.Goliad was dominated with Bacillales and Burkholderiales. In contrast, the APCNWR post-release samples Post.414/451.APCNWR and Post.415/455.APCNWR had a preponderance of Lactobacillales by at least 60% of the
cumulative abundance. Most notable was the Goliad post-release sample Post.434/486.Goliad with 88% Burkholderiales, which may be linked to the difference in release site.

Figure 3.23. Microbial community profiles of the cloaca of the pre- and post-release APC samples at the phylum taxonomic level. Samples were labeled by pre or post (release), both sample numbers indicating the same bird, and for post-release samples release site of APCNWR or Goliad. The last barcode included pooling of samples 414, 415, and 434 for the “Pre” group and samples 451, 455, and 486 for the “Post” group. “Other” included phyla Acidobacteria, Armatimonadetes, Cyanobacteria/Chloroplast, Gemmatimonadetes, Spirochaetes, Tenericutes, and the candidate division phylum TM7, each comprising less than 1% cumulative abundance in all samples. Samples Post.414/451.APCNWR and Post.415/455.APCNWR refer to individuals released to APCNWR while sample Post.434/486.Goliad refers to the individual released to Goliad.
Figure 3.24. Microbial community profiles of the cloaca of the pre- and post-release APC samples at the class taxonomic level. Samples were labeled by pre or post (release), both sample numbers indicating the same bird, and for post-release samples release site of APCNWR or Goliad. The last barcode included pooling of samples 414, 415, and 434 for the “Pre” group and samples 451, 455, and 486 for the “Post” group. “Other” included classes Bacteroidia, Deltaproteobacteria, Erysipelotrichia, Negativicutes, Sphingobacteria, uncultivated Actinobacteria, and uncultivated Bacteroidetes, each comprising less than 2% cumulative abundance in all samples. Also, the classes for phyla that comprised less than 1% in the phylum analysis were not included in this class analysis. Samples Post.414/451.APCNWR and Post.415/455.APCNWR refer to individuals released to APCNWR while sample Post.434/486.Goliad refers to the individual released to Goliad.
Figure 3.25. Microbial community profiles of the cloaca of the pre- and post-release APC samples at the order taxonomic level. Samples were labeled by pre or post (release), both sample numbers indicating the same bird, and for post-release samples release site of APCNWR or Goliad. The last barcode included pooling of samples 414, 415, and 434 for the “Pre” group and samples 451, 455, and 486 for the “Post” group. “Other” included orders Aeromonadales, Legionellales, Neisseriales, Methylophilales, Pasteurellales, Pseudomonadales, Rhodocyclales, Vibrionales, Xanthomonadales, unclassified Clostridia, and unclassified Gammaproteobacteria, each comprising less than 2% cumulative abundance in all samples. Also, the orders for classes that comprised less than 2% in the class analysis were not included in this order analysis. Samples Post.414/451.APCNWR and Post.415/455.APCNWR refer to individuals released to APCNWR while sample Post.434/486.Goliad refers to the individual released to Goliad.
CHAPTER 4

DISCUSSION

Understanding the composition of the gastrointestinal tract (GIT) microbiota of Attwater’s prairie chickens (APCs) may be useful to improving the overall health and wellness of these critically-endangered birds. The importance of the gut microbiota to its host has been demonstrated in numerous studies over the last decade. The three basic questions investigated in this project were to determine if there are differences between the cloacal microbial communities of wild-hatched and captive-bred APCs, to determine a change in cloacal microbial composition of a captive environment to a wild environment, and to determine differences in cloacal microbial composition of APCs treated with fluoroquinolones early in development.

4.1 Comparison between Treated and Untreated Individuals from Pooled and Individual Barcoded Analyses of Captive-Bred APCs

Preliminary data from the pooled treated and untreated analysis suggested that fluoroquinolone antibiotics cause a disruption in the cloacal microbial community structure. The pooled analysis of 5 treated samples and 7 untreated samples demonstrated a difference between the alpha (richness) and beta diversities (abundance) in the two groups by semiconductor sequencing and confirmed via sequencing of individual clones. *Clostridiales* increased from 1.8% to 26.9% and *Bacillales* decreased from 52.0% to 18.4% relative abundance from the untreated control as determined by semiconductor sequencing of gene amplicons targeting variable region 5 of the 16S rRNA gene. This was verified through Sanger sequencing of the clones where *Clostridiales* increased from 6.25% to 50% and *Bacillales*...
decreased 68.8% to 25% relative abundance from the untreated control. In addition, 
*Enterobacteriales* increased 10-fold from the untreated control and one clone was found to 
match *Shigella/Escherichia*. The notable increase in *Erysipelotrichiales* from the untreated 
control found with semiconductor sequencing was not observed with any of the treated clones. 
There were no known pathogens among the members of *Clostridiales* identified in the clones. 
In the rarefaction curves for both pooled untreated and treated groups, microbial richness was 
shown to be significantly greater for the pooled treated group (*p* < 0.01, ANCOVA).

With the advent of multiplexing barcodes, the same 12 samples from the pooled treated 
and untreated analysis were re-sequenced individually in the first analysis, and a second 
analysis with a new sample set was performed after depletion of template DNA with the first 
set of samples. In contrast to the pooled analysis of the treated and untreated groups, the 
findings from two independent analyses did not demonstrate a difference in the cloacal 
microbial composition between the two groups. There were no patterns in relative abundance 
specific to treated or untreated individuals. In the first analysis, Firmicutes dominated all 12 
individuals with over 80% relative abundance, but one sample from each treatment group 
exhibited a similar increased abundance in Proteobacteria. In the second analysis, Firmicutes 
dominated 7 of the 10 individuals, and in the other 3 individuals Actinobacteria was either 
dominant or equal to Firmicutes. Unexpectedly, three of the seven untreated individuals (415, 
431, and 437) in the first analysis and one of the untreated individuals (595) in the second 
analysis exhibited a high abundance of *Clostridiales*; thus, findings here suggest the presence of 
*Clostridiales* is not exclusive to the treated individuals. For both the first and second analyses, 
the bulk of the membership in *Clostridiales* at the genus level was unclassified *Lachnospiraceae*,
unclassified Peptostreptococcaceae, unclassified Ruminococcaceae, and unclassified Clostridiales, all of which form endospores, are gram-positive, and considered commensals of the GIT [53]. In the second analysis, four of the five treated individuals did experience an increase in Clostridiales whereas only one untreated individual increased in abundance, but the untreated individual was the second-most abundant in Clostridiales. Thus, if the fluoroquinolone treatment did have an effect on the microbial composition in the APC, Clostridium spp. were well adapted to survive under such adverse conditions as antibiotic treatment. Another interesting result in the second analysis was the high overall abundance of Corynebacterium in the untreated samples (34.2% ± 28.3%) while an overall depression (8.6% ± 4.7%) in the treated samples. Others have reported occurrences of Corynebacteria in the avian GIT [54, 55], and antibiotic administration may be a plausible explanation for the shift to low abundance in the treated samples.

In addition to a lack of distinct pattern in relative abundance specific to treated or untreated individuals, there was no clustering of samples by each group in the phylogenetic tree using weighted UniFrac distance metrics. The microbial richness, exhibited in rarefaction curves, did not show a clearly defined grouping of curves by treated and untreated samples for either the first or second analyses. However, the PCoA did exhibit a clustering of the treated group of samples along a diagonal line in the coordinate plane. A lack of significance between the treated and untreated groups of individuals may reflect the high variability and indicates the need for a larger sample size.

Differences between the results in the pooled and individual analyses of treated and untreated captive-bred APCs may be due to several factors. First, the mode of antibiotic
administration in this study was subcutaneous (non-GIT) instead of oral. In most studies reporting a shift in gut microbial composition, the route of antibiotic administration is oral; however, Looft, et.al. (2012) suggest possible shifts may not be specific to route of administration after E. coli was found to bloom in the GIT after parenteral (intravenous) administration. Fluoroquinolones have been shown to cause a significant \( (p < 0.01) \) change in gut microbiota from healthy controls, but that change was reflected in human fecal samples 5 days after the start of therapy [21]. It is possible that enrofloxacin does not have a significant prolonged effect on the cloacal microbiota to the extent tested here. For example, the gut microbial community in mice was found to revert back to baseline after three weeks of ending therapy with vancomycin [56]. One way to test this would be to add a temporal component of sampling prior to antibiotic therapy, during therapy, and after therapy similar to Ferrer, et.al. (2013). Another factor that cannot be overlooked is the fact that all APCs reared in captivity were administered the sulfonamide antibiotic as a measure to prevent/treat coccidiosis and/or sulfadimethoxine-sensitive bacterial infections. To date, no researchers have reported gut microbial composition as a function of sulfonamide antibiotics. To minimize stress, the conservation management staff administered the sulfonamide in the water shared by the APCs in a pen as opposed to restraining each individual to administer orally. This may have affected the variation seen between treated and untreated individuals in this study even though efforts to minimize sulfadimethoxine as a variable were considered when selecting individuals for this study.

This study stresses the importance of investigating the gut or cloacal microbiota individually rather than pooling samples of the same treatment or experimental group. A single
sample with an overabundance in membership of one taxonomic group may act as an outlier if the other samples in the same group are relatively similar in abundance and may bias results in a pooled study, as may have been the case here. In addition, the low sample size in the number of clones for the pooled analysis may have masked the significance in the increased incidence of Clostridiales of the pooled treated group. Similarly, sequencing of individual clones does not have the depth and coverage of sequences as seen with NGS of individuals.

Although the mean number of days in survival post-release was found to be significantly greater in untreated individuals in the second analysis, it is difficult to make any conclusions; however, it does suggest the need for further analysis in survivorship of APCs post-release between treated and untreated individuals with a larger sample size and utilizing a Cox Proportional Hazards Model.

4.2 Comparison of Wild-Hatched and Captive-Bred APCs

The cloacal microbial composition was demonstrated to be different between wild-hatched and captive-bred APCs (Figure 3.21 and Figure 3.23). The evenness, described as abundance and measured in percentage of reads classified by taxonomic rank, between the two groups (wild and captive) was significantly different at the phylum level \( p < 0.001 \), ANOVA. Results of the comparison of the cloacal microbiota between the wild-hatched and captive-bred APCs paralleled what Xenoulis and colleagues determined in their wild and captive parrot cloacal microbiota study [57]. In this study, wild-hatched APCs had less diversity at the phylum level with OTUs belonging to phyla Firmicutes, Proteobacteria, and Actinobacteria whereas the captive-bred APCs had sequences classified into Firmicutes, Proteobacteria,
Actinobacteria, Bacteroidetes, and unclassified Bacteria. In contrast to the wild and captive parrot study where Firmicutes dominated the cloaca of wild-hatched parrots 84.5%, in this study the wild-hatched APCs had a preponderance of Proteobacteria (86.2% ± 3.1%) in their cloaca. Even though the captive parrots and captive APCs exhibit similar microbial community profiles at the phylum level, the relative abundances are different. In captive parrots, there was greater membership from Firmicutes (63.2%) than Proteobacteria (22.9%), but in the captive APCs the abundances for Firmicutes (41.2% ± 20.1) and Proteobacteria (44.4% ± 10.3%) fluctuated.

The differences between wild-hatched and captive-bred APC individuals may be attributed to the different environments, and it must be said that diet is a primary predictor of community structure [22, 58]. The APC diet fed in captivity differs substantially from that in the wild environment. The wild and/or natural diet has consisted of ruellia, perennial ragweed, doveweed, grasshoppers, beetles, and other insects [2] and in captivity they are fed an APC-specific kibble made by Purina®. In addition, captive APCs are given the drug sulfadimethoxine in their water to prevent coccidiosis. Some other notable conditions in the captive environment include artificial rearing. For instance, once the APC hen lays her eggs, conservation management staff transfer the eggs into an incubator. After the captive chicks hatch, they are placed with a broody hen, which is a domestic chicken. As a result, captive-hatched chicks are not exposed to the bacteria of their mother and it is possible the degree of variation between individuals could be in part due to the lack of colonization of the chicks by maternally derived bacteria [59]. The differences in the ages between the wild-hatched and captive-bred APCs also likely explain much of the variation seen. The wild-hatched APCs were
approximately 28-42 days old while the captive-bred birds were 98.7 ± 4.0 days old at the time of sampling. Differences between findings in this study and other avian species are also likely attributed to conservation of microbiota among avian species as well as interspecies variation. As an example, the current captive APC population has been found to have decreased genetic diversity compared to historic populations [3] and the gut microbiome has been linked to genotype such that a mutation in a host sugar transferase gene altered the composition of the gut microbiota [60, 61].

4.2.1 Comparison between Wild and Post-Release

The evenness, described as abundance and measured in percentage of reads classified by taxonomic rank, between the two groups (wild and released) was significantly different at the phylum level \((p = 0.005, \text{ANOVA})\). The sampled individual that was released to Goliad, Post.434/486.Goliad, demonstrated similarities in cloacal microbiota at the phylum level with the wild-hatched birds from Goliad. The cloacal microbial community profile greater than 1% cumulative abundance for Post.434/486.Goliad comprised: Proteobacteria (91%), Firmicutes (6.7%), Actinobacteria (1.3%), and unclassified Bacteria (1.1%). In comparison, the cloacal microbial communities of the three wild chicks from Goliad comprised: Proteobacteria (87.3% ± 2.6%), Firmicutes (11.7% ± 2.3%), and Actinobacteria (0.8% ± 0.8%). The membership in the lower ranks within Proteobacteria diverged dramatically between the Goliad post-release individual and the three wild chicks from Goliad. For example, Post.434/486.Goliad encompassed 87.7% Burkholderiales, of class Betaproteobacteria, while the three chicks from Goliad had a mean of 0.7% ± 0.003%. In contrast, the wild chicks from Goliad exhibited more
diversity with the most abundant orders being Oceanospirillales (36.4% ± 0.1%), Enterobacteriales (27.6% ± 0.2%), and Alteromonadales (22.0% ± 0.1%), all of which are Gammaproteobacteria.

The single wild chick sample from APCNWR did not compare to the two captive-bred post-release samples, which were released to APCNWR (Post.414/451.APCNWR and Post.415/455.APCNWR). The wild chick was dominated with 82.8% Proteobacteria while Firmicutes was the predominant phylum for the two APCNWR post-release adults (75.4% ± 5.6%). Furthermore, the most abundant orders for the wild chick at APCNWR were Oceanospirillales and Alteromonadales where Lactobacillales and Burkholderiales were more abundant in the two APCNWR post-release birds. The predominant genera were *Halomonas* and *Shewanella*, both of which include members of characterized marine bacteria, and even halophiles in the case of *Halomonas*. While APCs are natural residents of the coastal prairie, the release sites of Goliad and APCNWR are approximately 40-60 miles from the Gulf of Mexico.

Differences between the three captive-bred individuals released into the wild and the four wild-hatched individuals may be attributed to their differences in age since it has been shown that the gut microbiota change temporally [62]. The wild-hatched individuals were approximately 28-42 days old at the time of sampling and the post-release individuals were approximately 219 (individuals 414/451 and 415/455) and 245 (individual 434/486) days old. In addition, stress as a factor influencing the diversity of the cloacal microbiota cannot be overlooked since it has been reported that stress causes a shift in microbial composition of the gut [43, 57, 63]. The drastic change from captive to wild environments, including the crating
and transport of individuals, as well as learning how to forage for food and ward off predators, is likely a stressful experience for the released APCs.

4.3 Comparison of Captive-Bred APCs Pre- and Post-Release

The effects of a change in environment on the cloacal microbiota between the three untreated individuals that were sampled prior to release and again approximately 179 days after release were demonstrated in this analysis. The evenness, described as abundance and measured in percentage of reads classified by taxonomic rank, between the two groups (pre- and post-release) was not significantly different at the phylum level ($p = 0.9$, ANOVA). Only two of the three pre-release samples, 415 and 434, contained members of Bacteroidetes and those members all but disappeared (from 8.6 and 11.2% to 0.01 and 0.10%) in the post-release samples, 455 and 486. At the order taxonomic level, the individual 414/451 exhibited an increase in the relative abundances of Burkholderiales (from 7.1% to 26.5%) and Lactobacillales (from 35.1% to 68.7%) post-release and a decrease in Bacillales (from 18.0% to 0.1%), Clostridiales (from 6.6% to 1.4%), and Oceanospirillales (from 23.0% to 1.3%) post-release. Similarly, individual 415/455 increased in Lactobacillales (from 10.6% to 60.0%), but the most notable difference was a dramatic decrease in Clostridiales (from 28.4% to 0.3%) post-release. Individual 434/486 exhibited a large increase in Burkholderiales (from 39.6% to 87.7%) and loss in Bacillales (from 34.1% to 1.0%), and it was the only individual to increase in Clostridiales (from 2.5% to 4.5%) post-release.

There were many differences in the cloacal microbial community profiles among the pre-release samples at all of the taxonomic ranks. Such differences among the pre-release birds may be a result of the three birds sampled placed in different pens during captivity and
therefore in contact with different birds. In addition, differences may arise if the chicks were placed with different broody hens, which are non-related domestic chickens. Stress may also be a factor for the differences among the pre-release birds.

As stated above, there was a notable increase in unclassified *Burkholderiaceae* in the post-release samples. Studies have reported *Burkholderia spp.* as a symbiont in the gut of insects such as ants and stinkbugs [64, 65]. While the conservation management staff try to supplement the captive APC diet with “natural” insects and vegetation, the primary diet is processed kibble. The APC diet will switch over to the “natural” diet of insects and vegetation once released into the wild, which, interestingly, includes ants and stinkbugs among others [2]. However, this does not explain why *Bulkholderiaceae* was not found to be as abundant in the wild chicks. Age is potentially a confounding factor with regards to the differences in age of the sampled wild-hatched chicks, as the microbial composition of the GIT in humans and other animals is known to change with time [66].

4.4 Other Factors Affecting Variation in this Project

4.4.1 Sample Collection and Location Variation

Cloacal swabbing was determined the optimal route for sampling since the critically-endangered APC may not be sacrificed or unduly stressed to obtain samples within the gut and it is not practical to obtain individual fecal samples from wild or captive APCs. Cloacal samples have been reported to at least partially represent the fecal and gut microbiota [57, 67] even though the cloaca is a common area for the digestive and urogenital tract for birds and may not represent the gut microbiota completely [68]. Most of the cloacal samples obtained and used
in this study were taken during an examination by a veterinarian at FRWC on the day APC individuals were transported to release location. Samples were obtained using a sterile cotton-swab applicator and inserted into the cloaca about the length of the cotton tip. While efforts were taken to minimize contamination and standardize the technique during sampling, it is possible that variation could have been introduced from different veterinarians and their cloacal swabbing technique. Furthermore, different conditions from those at FRWC existed at the release locations of APCNWR and Goliad, such as non-veterinary handlers and a less-sanitary environment. The most comprehensive method for sampling in order to accurately describe the gut microbial composition in APCs is to swab the epithelial layer of the gut directly or excise a cross-section of the gut to gain insights into the spatial organization of microbiota. Such a spatio-temporal study is forthcoming with the investigation and characterization of samples from euthanized APCs diagnosed with avian reticuloendotheliosis virus.

Another important factor that would improve the power of this study would be to increase the sample size. Sampling of APCs for the antibiotic analysis and the “pre-release” analysis was dependent upon the schedule of the captive-breeding program, and in general was designated on the days assigned for transfer to the release location. In addition, the list of individuals to be released was not finalized until the day of transport. For example, if particular individuals evaded capture by staff or if other birds were found to be unfit by the veterinarian, then the birds remained in captivity until another opportunity. Therefore, samples from all approved-for-release birds were collected and stored appropriately until medical records were reviewed after sample collection. Sampling in the wild or post-release locations of APCNWR and Goliad depended upon which birds happened to be captured for examination, and was
completely random. While the APCs are fitted with radio transmitters at the time of release, they record movement and not exact GPS location, and therefore it is not possible to find a specific individual of interest.

4.4.2 PCR and Sequencing Variation

Preliminary methods for this project included nested PCR, which is a method that employs two consecutive PCR reactions with two different primer pairs for the same locus within a gene. The first PCR is performed with an external primer pair and in the second PCR reaction the nested primers bind to the product of the first PCR reaction. This method has several advantages, such as increasing amplification specificity. It is unlikely that any nonspecific amplicons from the first reaction will have primer-binding sites for the second reaction, ensuring product from the second reaction will contain very little contamination from unwanted products including primer dimers, etc. In addition, it improves sensitivity by detecting species present in lower numbers through the amplification of ample amounts of DNA in the first PCR [69]. Previous results have demonstrated minimal PCR bias with nested PCR; however, nested PCR should be assessed with discretion as the use of two successive PCR reactions for the preparation of libraries for downstream sequencing can introduce greater PCR bias as a result of preferential amplification [69, 70]. For instance, if the first PCR excludes certain groups of bacteria, then only the groups represented in the first PCR reaction will be amplified in the second PCR reaction, which again may have limits of universality. At the start of the individual barcoding analyses between treated and untreated individuals, the PCR amplification protocol was changed from nested to a single PCR to minimize this as a source of
bias. The different PCR protocols employed in this project may therefore have technical variation between the different analyses. In addition, the preliminary stages of this project occurred soon after the release of the Ion Torrent™ PGM™ with 100bp chemistry. Throughout the project, sequencing technology improved such that the later analyses employed 200bp chemistry, and thus, manufacturer’s protocols changed.
REFERENCES


