INVESTIGATING THE DIFFERENCES IN HEALTH, PATHOGEN PREVALENCE AND DIVERSITY OF WILD BIRDS INHABITING SHADE-GROWN COFFEE PLANTATIONS AND FOREST FRAGMENTS IN SAN LUIS, COSTA RICA

by

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(Under the Direction of Ron Carroll)

ABSTRACT

This dissertation describes the differences in pathogen prevalence and diversity, as well as indirect health parameters of two avian communities in San Luis, Costa Rica. In the tropics, the balance between preserving forests and the ecological services they provide, and deforestation for human development is a major challenge. Sustainable agricultural practices, such as shade-grown coffee, have been promoted as areas that can act as forest surrogates, thus increasing available habitat for avian populations. However, their potential role as ecological sinks has not been studied. In this dissertation, I examine the potential role of shade-grown coffee plantations as potential areas where birds may harbor a higher level or diversity of pathogens than birds living in nearby forest fragments. I measured the level and diversity of a variety of directly transmitted (paramyxovirus, *Mycoplasma sp*.), vector-borne (blood parasites) and indirectly transmitted (e.g. endoparasites) pathogens in a community of birds inhabiting shade-grown coffee plantations and compared it to a community of birds inhabiting forest fragments. I also measured indirect health indices such as body condition and body mass. Some interesting findings include the presence of antimicrobial resistance pattern of the bacterial flora

in birds inhabiting sun-grown coffee plantations, but not found in shade-grown coffee plantations or forest fragments. Additionally, there were differences in the level of infection with *Haemoproteus sp.*, a blood parasite, in the White-eared Ground-sparrow (*Melazone leucotis*) by habitat type, such that individuals of this species living in shade-grown coffee plantations had a higher prevalence of *Haemoproteus* than those living in forest fragments. Finally, I propose a variety of mechanisms that could lead to this difference, one which is the introduction of backyard chickens, and explore the pathogen prevalence of backyard chickens in the region. I also examined the avian community composition of birds captured in shade-grown coffee and forest fragments and noted that the overall trends implies that forest obligate species colonize shade coffee at lower rates than forest fragments, and do not persist in coffee to the same extent as in forest fragments. Forest obligate species had lower interseasonal persistence in shade coffee plantations than in unfarmed forest fragments.

INDEX WORDS: Costa Rica, Neotropical bird, shade-grown coffee, forest fragmentation, disease, pathogen prevalence, endoparasite, ectoparasites, hemoparasite, community composition, virus, bacteria.

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DEDICATION

This thesis is dedicated to the people of San Luis, Costa Rica, who have taught me the beauty of laughter, generosity, humility, and happiness in simplicity.

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Any thesis is the product of the support of a variety of individuals, each of which plays an integral part in seeing this work to the end. This thesis, in particular, required the expertise of many to get at the “larger” questions, and their contribution deserves all of my appreciation. The order in which they appear is not as important as the tremendous support they all provided. Invariably I will inadvertently omit someone, and for that, I apologize in advance. Firstly, I am thankful to both of my parents for always encouraging me in all of my never-ending academic endeavors and for instilling in me (unknowingly, I am sure) a solid respect for wildlife and ecosystems. From snails, crabs and wax worms in a box, to rats, doves, turtles, goats, and everything in between that they allowed me to house, to supporting all my crazy travels to faraway places to develop the passion I have for wildlife disease investigation and preservation of our dwindling nature. Secondly, my husband Steve deserves a great deal of gratitude for all the grief I have caused him. Only married people can truly appreciate how much a spouse suffers through this process, and Steve, always believing in my professional growth, was incredible. I can confidently state that had it not been for my major advisor, Ron Carroll, I would not have entertained this work. It is only now, as I near the completion of this project, that I truly understand how his unique vision for the field of ecology, is the only reason I am here. His unwavering faith in my abilities, and his academic and moral support was invaluable. I have rarely followed the “traditional” route to anything, and where everyone else was asking “what is a veterinarian doing studying ecology”, he got it. When I felt insecure about this hybrid, he reassured me. He also provided a model of someone who flawlessly managed to

integrate research, conservation and humanitarian work. He is an inspiration and I look forward to many years of collaboration with him!

The rest of my committee was a great source of strength throughout my embryonic development. Dr. Pej Rohani's "tough love" at the beginning of my development got me "off my duff" and pushed me forward. Dr. John Fischer, and behind him, the entire SCWDS team, inspired me to "reach" beyond the current wildlife disease "grasp". Dr. Bran Ritchie taught me not to be scared to hack a new trail into unknown territory and always met me with a smile. Dr. Bob Cooper taught me the best course I have taken in my entire and lengthy career (Experimental Design). He is true to his love of teaching, and it shows. He always treated me like a colleague, something that gave me the strength to move forward in academia! Dr. Susan Sanchez provided me with a daily home, and while patiently teaching me valuable laboratory skills, made me part of her lab family. I will forever hold her on a pedestal--a scientist who manages a huge service appointment, and still produces solid, well-respected research, all the while gently and happily mentoring a large number of students. Her support went above and beyond what anyone expects of a committee member. I am so glad I had the opportunity to get to know you all and once again, THANK YOU!

At UGA several other people were intimately involved in my everyday work. Dr. Pedro Villegas was my expert collaborator on poultry, and provided encouragement and always a smile. He is a true-born teacher and mentor. MariCarmen Garcia and Sylva Riblet (as well as all the other folks in the Garcia lab) were instrumental in helping me realize the Mycoplasma work. MariCarmen opened her lab to me, Sylva held my hand through PCR, and the rest of the graduate students in the lab, experts themselves, never

laughed at me. Harmony Seahorn at the Poultry Diagnostic and Research Center Diagnostic Laboratory will probably delete my email address now that I am done. She provided fantastic help with hemagglutination inhibition assays. Natasha James provided laboratory assistance in the bacteriology lab. Dr. Michael Yabsley and Dr. Barry O'Connor provided all the parasitology assistance. Michael not only was prompt, but became a great friend along the way! Finally, my collaboration with Dr. Brady Mattson was integral to the chapter on community dynamics and he did it all with patience and integrity. In fact, a whole host of faculty at the Warnell School of Forestry and Natural Resources, mainly Sarah Schweitzer, John Carroll, Bob Warren and Michael Mengak were extremely supportive of my growth into an ecologist-veterinarian. I am sure we will all work together again.

The Odum School of Ecology (to me, always The "Institute") and all its players provided a fantastic environment to grow. The way the School values graduate student instruction permeates in everything they do, and I hope this never changes. The staff is an incredible, caring group: Misha Boyd, Janice Sand, Patsy Pittman, Anisa Jimenez, Jeremy Sanderling and all the others that were constantly bothered with my confusing requests deserve major thanks. My ECO-cohorts, always on the other end of the listserve ("Help needed from Sonia") are a fantastic group of people whose passion for ecology was infectious. Collectively, this group never judged me for being "the odd duck" and always impressed me with their professionalism and care for the Institute. There were some colleagues, that although they also count as having provided me with friendship and support, through endless conversations about my work, significantly affected the way I looked at Ecology, particularly Valerie Peters, Nicole Gottdenker, Scott Connelly, Alex

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This work was funded through a patchwork of sources. In the beginning, the Tinker Foundation Travel Award, awarded through the UGA Latin America and Caribbean Studies, was the only way I made it to Ecuador to explore and develop the first tentative questions. Had it not been for a variety of businesses (Neurocare Consultants in Palm Beach, Florida and Bankers Equity, Centurion Poultry, HOPE Animal Medical Center, and Jittery Joe's in Athens, Georgia), I would not have realized the first phase in Ecuador. I received two Sigma XI Grants-In-Training and a Sheldon Memorial Award for which I am extremely grateful. Sadly, due to the logistics of this project, I had to switch field sites, but I was fortunate to enjoy support initially through a UGA Teaching Assistantship, and later the federal government took a gamble on me through a National Science Foundation Graduate Research Fellowship, which finally allowed me true freedom to pursue my work in Costa Rica.

Anyone who has realized a project on an international location knows that it cannot happen without the coordination and support of so many. In Ecuador, Juan Carlos Unda and Pancho Prieto, two of the most impressive people I have ever met, were instrumental in helping me liaison with the Marianitias community, teaching me the beauty of highland Ecuador and teaching me the true meaning of overcoming adversity. The rest of the Maquipucuna Foundation, especially Rebeca Justicia provided logistical support with patience and I appreciate that they understood why I had to "abandon" them. In Costa Rica there are so many! Associate Provost of the Office of International Education Judith Shaw, provided that initial support that went on to become integral to my work. Quint Newcomer and Page McCorkle, from the UGA Costa Rica office were always willing to help, and I hope they understand how important all those little things they did for me were. Drs. Danilo Leandro and Randall Arguedas from the Simon Bolivar Zoo and the Ministry of Agriculture answered hundreds of emails, helped with equipment, transport and so much moral support. Dr. Danilo has seen me grow from a stubborn, know-it-all vet student (whom he appointed to cleaning cages for good measure), to, I hope, a humble researcher, and he is partly responsible for that transformation through all of his support. Carlos Jimenez, the Director of the School of Veterinary Medicine at the Universidad Nacional, and a collaborator on this project, was an invaluable source of "all things virological", as well as my main contact at the UNA. The ENTIRE staff at the UGA San Luis Research Station in some way or another contributed to my success. The ladies in the kitchen (Lupe, Sylvia, Alba, Lorena, Daisy, Xenia, etc) provided fantastic food infused with laughter, music and hugs. The maintenance staff was always happy to help with all my strange requests. The

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my thinking, and who lent me his eyes to examine the Monteverde Region and the folks who live there.

And speaking of field assistance, several UGA students participated in some aspect of this project: Avery Tomlinson, Jessica, Kate Spears, Raj Joshi, Paul Scarr, Andrea Smith, Ryan Malloy and Jason Norman joined me in the tiresome 12-hr days, the rain, cold, wind, mist and long hours of waiting for birds to hit the nets. They all brought laughter and a passion for fieldwork. I also instructed a large group of Biology majors through the UGA Teaching Assistantship and hundreds of veterinary students hungry for non-traditional animal medicine. They challenged and inspired me, and the opportunity to teach them, and all the other students I mentored during this time, was one of the most valuable experiences in this process. Logan Weygandt was a special case, since he continued the project for an International Fullbright Fellowship and gave me the opportunity to get my “San Luis” fix when I was writing the thesis and going through withdrawal. I wish I could say I was as diligent, organized and thoughtful when I was 21. He went on to become a collaborator and the better part of chapter 5 would not exist without him. I am sure we will continue to work together in the future. Jill Parsell, at the time employed by UGA San Luis Research Station, but later my field assistant, was there at the beginning of it all and transferred her love and enthusiasm (and technique) for birds in San Luis to me through those first and difficult field seasons. I cannot wait to work with her again.

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FORWARD: A TALE OF TWO DISCIPLINES

It is my belief that the scientific community dedicated to the study of wildlife disease, is at the cusp of significant changes. Increased coverage by the media, recurrent reports of emergent diseases, threats of bioterrorism, the socioeconomic importance of some pathogens, the increase in international movement of pathogens, greater emphasis on spatial dynamics of populations in ecology, etc have all resulted in an upsurge of interest in the study of wildlife disease. As a result, there are more scientific disciplines now engaged in this field. This, of course, leads to a more diverse group of people, trained in a variety of ways, approaching problems from a range of perspectives and has led to some elegant work. Unfortunately, however, this also means that the different disciplinary groups, having "grown up" in their own, often insular, academic environments, can, at times, have trouble communicating. One way to remedy this issue is to produce scientists who receive training in a variety of disciplines and who thus learn a variety of "languages", research protocols and bodies of knowledge. Such was my career path. I was a budding zoologist when I was admitted into veterinary school. In fact, my undergraduate advisor found it a "shame" that I should go on to receive, what he called "technical" training, rather than forge forward in graduate school. But "I had a dream…" to apply that which I had learned in my field wildlife courses to work with free-ranging animals. Upon entering veterinary school, my idea of working with wildlife was undefined. I quickly fell into the clinical medicine track because the most common t t

type of experience with wildlife in a veterinary school is diagnosing, treating and rehabilitating injured animals. I made every effort to acquire as much non-traditional animal medicine experience as possible, until, at some point during the second year in my residency training (and now 3 yrs out of veterinary school), I realized I had fallen away from my original goal. Whereby I was learning fantastic diagnostic and treatment techniques for working with individual wild animals, I was not moving much further in understanding why, for example, in the wild, certain animals become infected and succumb to disease, while others do not. At about this time, I was fortunate to be involved with the Proyecto Danta (Tapir Project; http://savetapirs.org/) in Costa Rica and the themes of the wildlife-domestic animal interface, fragmented and artificially concentrated populations, the management of declining populations, genetic impoverishment, and fractured communities all began to swirl around my head. As I led the first investigation of the diseases of a free-ranging population of tapirs, I began to consume any information that would help me understand if and how disease might be playing a factor in affecting this population of animals. The wildlife medicine literature was full of papers describing the prevalence of pathogens in this or that other animal, but something was missing from all those papers: So what? What does it mean if you find evidence of the occurrence of this agent or another? How is it affecting the population? (Deem et al 2004; Uhart et al, 2003; Rosetti et al, 2003; Karesh, et al). I do not mean to be critical here, those papers are paramount baseline information, which can prove useful in developing hypotheses if an outbreak occurs and they fill in the gaps of knowledge we have about disease in wild populations, especially in those that are declining, but they did not satisfy the question—where does disease figure in with all the other factors that affect

and regulate populations and communities of animals? At the same time, a group of papers began appearing that approached this very question from a theoretical standpoint. While veterinary medicine was busy describing, for example, *Chlamydia psittici* as the causative agent of infertility and keratoconjunctivitis in koalas, others were taking an intriguing approach with approaches such as Augustine's model of Chlamydia transmission dynamics (Augustine, 1998).

While I was learning how to handle and anesthetize elephants, kangaroos, skinks and tapirs, how to determine a list of differential diagnoses and common diseases for each group, or how, reptile and mammalian immunology compare, I still could not answer what it meant, for a population of tapirs to find antibody evidence of infection to viral diseases. Standard veterinary texts concentrated on agents and the pathology they caused but not on the changes to population dynamics that might result from the infection. Epidemiology texts informed me on transmission mechanisms and disease spread, but they did not take into account the natural history of the animals (in the case of tapirs, solitary, nocturnal, shy creatures) and always dealt with disease in the context of its need to be managed or eradicated. In 2001, while studying for the American College of Zoological Medicine Board Examination, (for which I had selected "wildlife" as my subspecialty), I picked up Gary Wobeser's book on wildlife diseases of wild animals and everything changed. Regardless of the fact that he was a pathologist and trained to look at host-pathogen relationships classically, a few sentences in the book's introduction and concluding chapters cinched it for me (Wobeser, 1994). He speaks of turning towards a more holistic approach to wildlife disease, a process, it seems, he had gone through himself. In the preface of his latter book, he states:

"……my interest…became concerned more with general aspects of health in wild animals, such as how and why various diseases occur in wild animals, why animals and parasites appear to get along better in some situations than in others, and the effects of disease on populations rather than on individuals. There is a growing realization on my part that disease is one ecological factor among many and that disease can never be considered satisfactorily in isolation." (Wobeser,2006).

By the time Dr. Wobeser published that second edition, I was deep into my PhD, the subject of this thesis. The only answer I could find to my inadequacies of perspective was to seek another degree. I feel vindicated now, as the field of disease ecology is growing exponentially, and veterinary schools across the country are incorporating conservation medicine and wildlife disease investigation with an ecological perspective into their curricula. In fact, in a recent publication in the Journal of Veterinary Medical Education, Jonna Mazet stated

"It is time that we integrate ecosystem health into our curricula to nurture and enhance an expansive way of looking at veterinary medicine and to ensure that veterinary graduates are prepared to excel in this new and complex world, in which the health of wildlife, domestic animals, and people are interdependent." (Mazet et al, 2006).

Perhaps the reason why "conservation medicine", (a relatively new discipline that focuses on the intersection of human, animal and ecosystem health) has gained so much attention recently, is because it both differs from traditional wildlife disease investigation by being most concerned with declining populations of animal species, (versus the traditional focus on managed and game animals), or those that may pose a risk for humans, but most importantly, because, as a result of working with declining, small,

fragmented, endangered populations and communities, it is *forced* to incorporate the principles of the science of ecology .

We can get lost in semantics, trying to define the ecosystem health practitioner vs. the conservation medicine practitioners vs. the wildlife disease investigator, but the point remains, we are moving towards a more holistic approach to wildlife disease investigation and management—and the current state of affairs of the world demands it. In some cases, we no longer have the luxury to sample or sacrifice thousands of animals to describe their pathogen loads, or wait until an outbreak occurs. Landscapes are changing rapidly, habitat loss is unprecedented, and population declines are becoming the norm. Disease is but one of many factors that affect populations and now, more than ever, is the time to look at the big picture.

We have some growing pains. This is the topic of a paper my colleague, Nicole Gottdenker, and I are writing, exploring all the painful aspects of this new growth (Hernandez-Divers and Gottdenker, in prep). For example, there are still many who are not classically trained in disease or ecology and, dabbling in fields outside their expertise, risk major criticism for not adhering to basic principles or misinterpreting information. In some cases, there seems to be a parallel growth of wildlife disease investigation that fails to cross-pollinate, such that one group completely ignores a body of literature that the other finds paramount, or worse, adopts some, often misinterpreted, portion of the literature and not others. There are intellectual, ego-based territorial battles (“we are the wildlife disease experts”) and I have seen well-respected professionals quickly reduced in my opinion because of their lack of respect for others in related fields. I propose that the main problem standing between ecologists and veterinarians is simply perspective.

Ecology deals with trends, and associations. It aims to describe general rules for the purpose of explaining ecological evolutionary forces, and making predictions. Take the theory of island biogeography and its application to studying habitat fragmentation as a prime example. Veterinary medicine is all about details. As veterinarians we easily become entangled in details, often quoting exception against exception to the rules. It takes some mental calisthenics to let go of the details and start grasping towards trends. I used to mistake this for compromising technique, or project design, but that need not be true. In this world of specialization, where everyone takes pride in being "really good at one little thing", it is hard to stretch out and generalize. But the answer to all the above maladies is simple—collaboration. Truly multi-disciplinary approaches in wildlife disease projects involve all players not as mere technicians that "do" one part or another, but rather engage all in the intellectual process of hypothesis formulation and project design. This also means collaboration at the organization level, such that journals should include members of other disciplines as associate editors, and conferences should be open to sessions that are populated by members of related, yet different sciences. Of course all of this involves human nature, trust and letting go of some control, issues so complex I cannot delve into them here. But suffice to say, this is an exciting time to be a team player in melting pot of disciplines, and I cannot wait to see what emerges.

Had I aimed for a PhD in wildlife disease, my project might have been called "*The Parasites of Wild Birds in Shade-Grown Coffee Plantations*", or "*Transmission dynamics of paramyxovirus between Gallus domesticus and Catharus aurantiirostris*". Both projects would have had merit and would contribute to understanding wild bird diseases, but either through descriptive or experimental methods, I would have focused

on the host-pathogen relationship. Instead, the title has become "*Investigating the differences in health, pathogen prevalence and diversity of wild birds inhabiting shade-grown coffee plantations and forest fragments in Costa Rica: Are Shade-grown coffee plantations disease sinks for wild birds?*" and the aim in describing the difference is not only to determine if the birds in these plantations have a higher prevalence and diversity of pathogens, but to investigate the relationship between human-imposed effects to a landscape and disease dynamics. In other words, in our efforts to create forest surrogate habitat (such as shade-grown coffee), have we ignored some basics (such as aggregating birds in small areas, and placing them in ever increasing contact with domestic animals and humans) that offset the benefits of this habitat? Such is the question and it, perhaps, has a broader application than the aforementioned perspective. With this mindset, I set forth to present this dissertation. Enjoy.

CHAPTER 1

LITERATURE REVIEW:

SHADE-GROWN COFFEE--CONSERVATION VALUE AND RISK FOR WILD BIRDS

Habitat fragmentation and Disease

Habitat loss for wildlife has reached unprecedented proportions and is now the leading cause of species extinction (36). In particular, habitat loss affects 89% of threatened birds and of those birds affected by habitat loss, 74% are immediately impacted due to loss of tropical forests (36). In the tropics, the balance between preserving forests and the ecological services they provide, and deforestation for human development is a major challenge. The New World tropics are one of the regions with the highest deforestation rates, estimated at 13 million acres of forest per year (2, 104). The categorization of biological "hotspots" in 2000, which highlighted Central America's value as an area of conservation priority, should have mobilized efforts to further protect biodiversity (63). While deforestation rates of natural forest have dropped considerably since the 1990's in Costa Rica, its remaining forests still face threats from illegal timber harvesting in protected areas and conversion for agriculture in unprotected zones (45). Tropical deforestation was also strongly supported by national and international loans that promoted increased cattle production (54).

The increased edge effect and smaller habitat patch area that results from habitat fragmentation poses a variety of threats to biodiversity, including**:** 1) promoting biological impoverishment as small populations disappear, 2) creating favorable conditions for the persistence of exotic species, 3) favoring the abundance of generalists species and 4) potentially creating points of artificial aggregation or concentration of wildlife. Some of these phenomena have recently been examined in terms of their relationship with an increase in diseases in wildlife populations. Although ignored for many years, disease has now been recognized as playing an important role in natural systems, often altering ecological communities (3, 5, 19, 23) . In the last 20 years, it has become clear that disease poses a serious threat to natural populations, especially those that are endangered, occasionally leading to significant declines or extinctions and examples abound in the literature (4, 12, 42, 46, 65, 102, 109). Examining the potential mechanisms involved with habitat fragmentation, and how it might promote disease risk should prove useful for formulating preventive and management policies.

Biological impoverishment--Central to ecology is the ‘‘insurance hypothesis’’ (28, 64, 70, 110) , which holds that biodiversity stabilizes functional properties of communities to environmental perturbations, especially in environments with many specialized species. Similarly, describing the relationship of disease dynamics, the concept of the “dilution effect” emerged, to describe how biological impoverishment could change the prevalence of infectious diseases. The inverse relationship between the biodiversity of intermediate hosts available for Ixodid ticks and the rising prevalence of human Lyme disease cases in North America is the most notable example for this hypothesis, but other studies have

explored it with other diseases, such as *Trypanosoma cruzi* (51, 103). In these cases, disease increased as biological diversity of hosts decreased. Given that the Millenium Ecosystem Assessment estimated that the distribution of species on Earth is becoming more homogenous, this may have important consequences in the near future (2005).

Edge effect and habitat heterogeneity--Compared with edge effects in large habitats, the edges of habitat fragments extend external threats over a larger fraction of the habitat. In doing so, these edges create regions that subject avian populations to a variety of pressures, some examples of which include physical effects from increased wind, sunlight and temperature, higher rates of predation and brood parasitism , all of which result in lower nest success, (38, 72, 106). Metapopulation analyses have suggested that corridors, inherently composed of a large "edge" area, may play a role in increasing disease transmission (35) and should be investigated as a trade-off against the putative beneficial effects of corridors. Although to our knowledge no study has explored what infectious disease risks associated with edges might pose for avian populations per se, it would be easy to hypothesize that these boundaries are zones that may harbor higher numbers of intermediate vectors (due to microclimatic conditions and subsidies from agricultural hosts), and/or promote more contact between wildlife and domestic animals. For example, the presence of domestic dogs in and around protected areas and the threat they might pose for the conservation of endangered carnivores through disease transmission has received a lot of attention from the conservation communities (13, 14, 25, 105). In habitats where forest fragments are surrounded by agricultural land, we would expect similar issues would apply to birds where large populations of agricultural

and peridomestic birds support large populations of pathogens and vectors that could "spill over" into bird populations in fragmented forest habitats.

Introduction and Persistence of Exotic Species--Both malaria and avian pox virus exemplify the introduction of a variety of exotic organisms into the Hawaiian islands and which are responsible for the decline of the endemic avifauna (102). The dwindling populations of Hawaiian birds forced some elegant research elucidating how deforestation and habitat fragmentation have decreased available habitat for endemic avian populations, increased anthropogenic habitat for introduced birds that serve as hosts for many pathogens and allowed the establishment of introduced mosquito vectors in regions where these endemic birds live (88, 101). The story of Hawaiian birds is, however, complex. Disease risk was influenced not only by habitat fragmentation but also because the altitudinal and weather gradients that play a role in vector biology delineated a boundary of disease. Because endemics declined within the disease boundary but have persisted outside the boundary, the important role of disease was apparent. This point, the primary role of a disease introduced by an exotic species in a population or community decline, is not always crystal clear and often requires further investigation to disentangle other contributing environmental factors.

Artificial aggregation/concentration, supplemental feeding and Disease of wildlife-- Artificial aggregation or concentration of animals in a small space promote the transmission of infectious diseases by ensuring both direct contact at a higher rate than "normal" or, in some cases, creating areas heavily contaminated with bodily secretions

and excretions (e. g. feces). Furthermore, because population concentration implies attractive resources (or fewer alternative resources) the rate of susceptible individuals coming to the resource may remain high. Although obvious to most epizootiologists, events connecting aggregations of animals and disease emergence have just begun to gain coverage in the more general ecological literature. Specifically, the connection between winter feeding stations and the establishment of *Mycobacterium bovis* in native white tail deer populations in Michigan (while also allowing more frequent contact with cattle) and the relationship between concentration of house finches at birdfeeders and the spread of *Mycoplasma* conjunctivitis have brought prominence to this issue (20, 34, 87) . In the latter example, it was originally suggested that tube birdfeeders that required the insertion of the bird's head into it could act as important fomites (vehicles for transmission) for *Mycoplasma* transmission; however, a later study failed to prove the significance of this mode of transmission. Nonetheless, the concentration of these birds at food sources provides bird-to-bird contact at a higher rate than in natural feeding areas, a fact that was not disputed (20). Several other examples are in need of further examination, such as whether bird feeders and urban ponds are also responsible for increased prevalence of *Salmonella* in wild passerines and waterfowl (15, 100). In a landscape where food subsidies attract birds at high concentrations, transmission of infectious diseases might be facilitated. It has long been a concern of wildlife managers that the decrease of available wetlands translates to concentration of more birds in fewer sites, which could be particularly important for wetlands where, for example, avian cholera or botulism outbreaks occur periodically (10). This should be especially true during periods of migration when the rate of arriving susceptible individuals is high.

Promoting Generalists Species--Fragmented, biologically impoverished landscapes tend to promote the persistence of generalist species (66). By "generalist species" I refer to species that are found in a broad array of habitat types, especially disturbed and anthropogenic habitats. In the context of my study this is important because generalists can play important roles in disease emergence, persistence and pathogen transmission. It appears that species that utilize anthropogenically disturbed habitats, (e.g., raccoons, crows, grackles, etc.) coincidentally also tend to be more competent pathogen hosts and in high abundance, thereby providing great sources for pathogen colonization and multiplication. For example, in the case of West Nile virus in the Western USA, certain avian host species, such as Western scrub jays, *Aphelocoma coerulescens*, exhibited the highest levels of viremia, followed by house finches, *Carpodacus mexicanus*, and house sparrows, *Passer domesticus*. Of these, house sparrows, considered generalists of high abundance, do not die of WNV infection and are likely to play a more important role in the spread of the virus than other species (82). In addition, these species are highly mobile, making a more diverse use of the habitat and moving through different habitat patches, thus facilitating the movement of pathogens. Landscape foci that provide regions of contact between wild birds and an enhanced pool of generalists could contribute to an increase in disease prevalence.

It is clear that habitat fragmentation, through a variety of mechanisms, can lead to disease emergence, or promote pathogen introduction and transmission. On the other hand, forest fragments are, by definition, isolated. For less mobile species, vectors, or for habitat specialists, forest fragmentation may actually reduce pathogen transmission.

Disease and Avian Populations

In 2001, Friend pleaded with the avian scientific community to focus more attention on how disease has affected avian populations in recent years (26). He discussed "*two barriers inhibiting adequate response to disease emergence in avian and other wildlife species are 1) the persistence of perspectives that disease is not a significant factor relative to the population dynamics of wild species; and 2) a tendency to focus on the affected species or the causative organism rather than on the affected environment.*" In other words, he argued that for a fuller understanding, disease should be studied in an environmental or ecological context not just from a medical treatment perspective.

The field of avian disease investigation has been hampered by the same factors that limit wildlife disease investigation in general, such as a lack of basic population ecology (hampering efforts to apply epidemiologic theory), problems related to a lack of data on population numbers or community composition, challenges associated with sample collection, obstacles related to the diversity of wild animal communities and within-species diversity of pathogen strains, challenges in understanding the relationship between and among diseases and other factors, such as predation, difficulties related to the relationship humans have with wild animals and, finally, issues with acquiring funding specifically to study diseases in wild animals (108). Most of the notable and most researched examples of avian diseases in wild birds have dealt either with events of highly visible mortality for which detection of dead animals by the public has driven investigation, (e.g. West Nile virus in crows, Newcastle in cormorants, *Mycoplasma* causing conjunctivitis in house finches, and regular outbreaks of avian cholera in waterfowl) or, in cases of population decline, especially of endangered species (eg.

malaria of Hawaiian endemics, avian vacuolar myelopathy in Bald eagles, botulism in White pelicans). But it is important to remember that even diseases which cause high mortality in abundant species often go unnoticed. For example, Stallknecht details that during a hemorrhagic disease outbreak in white tailed deer, an 8% mortality was estimated based on mortality monitoring of radiocollared individuals, yet not a single case was reported by the public (95). Aside from highly visible diseases, little is understood about the more subtle population effects that other pathogens can cause, such as the effects disease might have on reproductive failure, decrease in recruitment or indirect mortality caused by increasing the effects of other stresses or limiting the ability to escape from predators. Futhermore, and particularly in the tropics, little research is dedicated to elucidating diseases of common, resident species, often overlooking that these species are often also competent pathogen reservoirs and highly abundant.

Chickens as reservoirs and sentinels of avian pathogens--With a global distribution, the backyard chicken is probably the most common exotic bird species purposely introduced in the world and may be a source of pathogen transmission to wild birds. Several investigations support the correlative theory that wild birds may play a role in the transmission of pathogens to chickens (17, 52, 73, 74, 86, 91) . However, research into the details of pathogen transmission between free-ranging backyard chickens and wild birds is scant (30, 31, 55). Although, for some pathogens, such as avian influenza, and *Mycoplasma* conjunctivitis, a relationship has been established connecting pathogen transmission between chickens and wild birds (21, 32, 98, 99) . This may be influenced

by economics—human and production animal health research is better funded than wildlife disease and small scale farm investigations.

Chickens are often used as sentinels to monitor the presence and prevalence of certain infectious diseases and in most cases are considered good indicators of avian disease agents in a given environment (44, 62). In my study, chickens were used to determine the avian pathogens present in the San Luis valley ecosystem. Because chickens and wild birds co-occur in shade-grown coffee plantations my goal was to determine if domestic birds pose a risk for wild birds that share these habitats.

Conservation of Costa Rican avifauna

Costa Rica is famous for its high biodiversity and commitment to conservation. Specifically, the Monteverde region, in the Northwestern part of the country and the location of my study, is the second top tourist destination, attracting ecotourists seeking its natural beauty and rich avian biodiversity (Costa Rica Tourism board, 2008). Therefore, preservation of avian biodiversity in this region should be a priority, both for economic and conservation value. Costa Rica houses an incredibly diverse avifauna, with more than 840 species (96) . Aside from its aesthetic and spiritual value, the maintenance of this biodiversity is important for functions that birds provide, such as pest control, seed dispersion and ecotourism, the latter of which is a $1.9-billion-a-year industry in Costa Rica, (U.S.A. State Dept 2007). Of the 840 species found in Costa Rica, 25% are migrants that spend the summers in North America, thus providing additional ecological functions on their breeding grounds, and supporting the US's $32 billion dollar bird watching industry (US Fish and Wildlife Service, 2001). The future of avian biodiversity

in countries like Costa Rica depends on a combination of conservation efforts, including sustainable agroforestry practices.

In a study of the native avian fauna of southern Costa Rica, Daily et al. found that 55% of the species occurred only in native forest, 23% of the species only occurred in the agricultural habitats, and 22% of the species occurred in both habitats (18). However, according to Stiles (1985), more than two thirds of Costa Rica's avifauna utilizes human-altered landscapes (including traditional polyculture plantations), as long as there are at least small forest patches or trees nearby. A recent publication examined the persistence of three forest bird species in the Costa Rican countryside, illustrating the important role of even small forest remnants in agricultural landscapes (90). Additionally, the conservation management of both the Resplendent Quetzal (*Pharomacrus mocinno)* and the Three-wattled bellbird (*Procnias tricarunculatus*) in the Monteverde region provide excellent examples in which coordinated efforts to manage human-dominated landscapes maximized habitat availability during the altitudinal migration of these species (80, 81). Thus, it is very valuable to examine how forest birds utilize human-altered habitats and the benefits and risks that these habitats present for birds.

Conservation of Avian Fauna through Shade-Grown Coffee Surrogate Forest

The development of protected areas is important, particularly for animals threatened by poaching or over-harvesting, for limiting habitat loss due to logging, and for highly specialized and endemic species (78). However, most wildlife managers understand that protected areas are not the panacea they were once thought to be, and that they are only one tool in the conservation toolbox (22, 49). New conservation approaches

differ in that they 1) emphasize human-dominated landscapes, 2) focus on ecosystem services, and 3) utilize innovative finance mechanisms (85). Since the 1980's the field of sustainable development and agriculture has gained much momentum to ensure that private land under careful management, while providing economic support for people, could be used to maintain habitat available to support biodiversity, as well as to buffer protected areas. I was particularly struck by a 1998 essay on "Gardenification" written by the eminent tropical ecologist, Dan Janzen, in which he suggests that "gardens will live forever", because intrinsically *people* care *for* them. He was using this concept as it applies to a type of management--habitat restoration (thus, people "tending" to gardens), but nonetheless, it offers an interesting perspective on a broader scope. I later heard him speak, where he utilized several examples of land restoration to suggest that we rename "the wild" (which no longer truly exists) to "the garden", which invites humans to participate, and protect while being intimately connected with, and invested in, the land (40). It was with Janzen's eyes that I first viewed the promotion of shade-grown coffee as a viable way to conserve useful habitat in Latin America. In some regions of Latin America, shade-grown coffee makes up the only remaining forested habitat. In northern Chiapas, about 90% of coffee is shade-grown (57) and in El Salvador, <10% of the original forest remains, but since 92% of the coffee is grown under shade, it accounts for 80% of the country's tree cover (67, 83). Coffee managed under a floristically and structurally diverse canopy provides important habitat for biodiversity (57, 68, 69). Shade coffee plantations can be viewed as "gardens", tended by small farmers for economic benefit, while potentially playing a vital role in maintaining biodiversity. Prices farmers receive for shade-grown coffee is typically higher and more stable than for conventional

"sun coffee", thus farmers have an incentive to produce coffee under tree canopies. Coffee production in Central America makes up 5-25% of exports, making this product economically important, however, vulnerable to market fluctuations (33). Costa Rica is the fifteenth largest exporter of coffee in the world and the fourth largest in Central America. However, 92% of the coffee produced in Costa Rica comes from parcels less than 5 ha in size (93). As a way to encourage farmers to produce coffee under systems which promote biodiversity and stabilize higher revenue for the farmer, a variety of certification types have been developed. Each measures a specific set of criteria, but aims to standardize a way for consumers to identify coffee that is grown in "environmentally-friendly" ways, while promoting fair wages for producers. Three types of certifications are available: organic, fair trade and shade. Shade coffee production is certified under two programs: Rainforest Alliance and Bird Friendly® (BF). Bird Friendly certification also requires organic certification. Fair Trade certification emphasizes equitable revenue for farmers. Organic certification restricts the use of pesticides and synthetic fertilizer.

Bird Friendly certification places the value of shade coffee on protection of the native avifauna. According to the Smithsonian Migratory Bird Center's Bird Friendly Certification program, there are two types of shade: rustic or planted shade. Rustic shade refers to coffee grown under native trees in old growth, or, more typically, secondary forest. In contrast, planted shade coffee refers to plantations of coffee, in which trees, native or non-native, are planted specifically for shade. In this system, two native tree genera, *Inga* and *Erythrina*, are most commonly used in Latin America. In either system, additional trees, shrubs, and other vegetation may be planted to increase the farmer's revenue and/or to enhance biodiversity. SMBC recommends a minimum of 40% cover be

maintained, without trimming or removal of epiphytic plants or hemi-epiphytic vines on the shade trees in both shade and rustic systems in order to maximize biodiversity (Smithsonian Migratory Bird Center, http://nationalzoo.si.edu/ConservationAndScience/MigratoryBirds/Coffee/). Furthermore, SMBC recommends that "backbone" shade trees (those making up the largest proportion of shade) be allowed to attain a minimum of 12-15 m in heigh and that shorter (for example, fruiting trees for human use) and taller trees (such as timber producing species) be planted in between the backbone species to increase vertical structural diversity. Lastly, living fences or border trees are recommended when plantations abutt roadways and agricutlural landscapes to intercept strong dry season winds and thereby prevent dessication of understory vegetation, and a buffer of secondary growth (5 m on each side) along streams is recommended as well. Figure 1 illustrates the structural complexity of these coffee systems.

The benefits to biodiversity from shade-grown coffee systems are well documented and have been summarized in recent reviews (57, 70, 75) one of which has outlined the specific benefits of shade certification to biodiversity (77). However, most studies focus on the diversity and abundance of specific species (ants, birds) and primarily in some specific country region (e.g. Chiapas highlands in Mexico) and few studies examine ecosystem functions provided by shade-grown coffee and how they compare with forested systems (70, 75, 76, 94).

Shade-grown coffee as ecological sinks: Prevalence and diversity of pathogens of wild birds in coffee vs. forested habitats

Given the above discussions, shade-grown coffee parcels in Costa Rica could be viewed as human-altered habitats that act as surrogates of natural forest and provide additional habitat for forest birds. However, these parcels may also pose a risk of pathogen transmission to birds that occupy them because: 1) the parcels are biologically more impoverished than nearby forest habitat, 2) are often small and consist of large edge effect, 2) promote the persistence of generalists species; and, 3) most important to this study, they typically harbor flocks of poultry, that are known to be potential reservoirs for avian pathogens,

Project Objectives and Hypotheses

Objective

The primary objective of this study was to investigate the difference in prevalence and diversity of pathogens of wild birds inhabiting shade-grown coffee parcels and wild birds inhabiting forest fragments.

Hypotheses

Null hypothesis: There is no difference in the health or prevalence and diversity of pathogens between the same species of birds living in shade-grown coffee and forest fragments.

Working hypothesis: The pathogen prevalence and diversity of birds living in shade grown coffee plantations is **higher** than those living in old growth secondary forest and thus health indices for these birds are **lower** than for forest birds.

Background: Absolute pathogen prevalence and diversity (that is, all of the potential macro and microorganisms that could affect the health of birds) obviously cannot be measured, and thus, I am limited to those that can be quantified is some way. The term "health" can be very difficult to measure. We recognize that "disease" (as "an impairment that interferes with normal function" (107) is also difficult to measure and that most animals exist in some continuum between "absolute health" and "death" due to disease (107). Thus most disease surveillance investigations can only measure disease in a specific context. I aimed to measure health (or absence of disease) as it is reflected by normal function; as absence of extrinsic factors (e.g. macro- and microorganisms); by quantifying the presence or absence of specific diseases or infections; and understanding that I did not measure some functions, which may be impaired by disease (e.g. reproduction). It should also be clear from the beginning that I did not set out to prove that individual birds might be dying, or populations of birds might be declining, as a result of either contact with more conspecifics (artificial aggregation) or with free-roaming backyard chickens. The purpose of this study was to create a model with which, adequately utilizing biomedical tools, one can investigate how disease dynamics change in human-altered systems. Thus, if I found more evidence of, for example, a virus in birds that inhabited coffee plantations, (when compared with same species that lived in forest fragments), even if that individual did not exhibit clinical disease, or even if the population did not *seem* in decline, I would consider this an important finding, because it is evidence of disease risk.

I further subdivided the hypothesis above into:

Hypothesis #1: Evidence of directly-transmitted infectious diseases, such as Paramyxovirus, *Mycoplasma* spp. and Infectious Bronchitis Virus (IBV), will be higher in birds living in shade-grown coffee plantations as a result of artificial aggregation and contact with *Gallus domesticus*.

Background: In choosing directly-transmitted diseases, viruses are the obvious choice, as they typically require intimate contact, which generally speaking leads to rapid transmission and infection, often leaving behind the evidence of humoral immunity (antibodies). Mycoplasmal diseases are also ideal, as their recent emergence in Passeriformes is well documented.

Paramyxovirus antibodies have been reported in a variety of wild birds, although clinical disease and mortality is most often reported in members of Galliformes, Columbiformes, Psittaciformes, Anseriformes, and Double-crested cormorants. There are currently 9 serotypes described, of which only AMPV-1 is recognized to cause disease in wild birds (47). The virus is shed in the feces, bodily fluids and eggs and can survive outside the host, making transmission via fomites more likely (e.g. feeders). The chicken corrals in San Luis, with a high amount of ground organic material, and both humid and cool climates, would support virus survival for >200 days (47). Most infections do not cause disease in wild birds, but experimental studies in cormorants, as well as virus isolation from a variety of apparently healthy wild birds, demonstrate long periods of shedding leading to infection of other birds, is typical. On the other hand, virus infection with common serotypes in chickens (called Newcastle Disease) causes respiratory, reproductive and central nervous system dysfunction which can rapidly lead to death. Population effects of Newcastle Disease Virus (NDV) have not been determined, even

for wild Rock Pigeons, where the disease has been well documented. One report of paramyxovirus in wild birds of Costa Rica exists (31).

Seventeen species of *Mycoplasma* have been identified in wild birds (53). Some types of Mycoplasma have only been isolated from specific species of birds (e.g. *M. corogypsi* in black vultures). There are two *Mycoplasma* diseases in domestic poultry of importance: *Mycoplasma gallisepticum* causes chronic respiratory disease and has been thoroughly described to cause clinical disease in wild turkeys and house finches. Within *M. gallisepticum* (MG), several strains exist, translating to a range of host trophism and diverse modes of transmission (53). *M. gallisepticum* is transmitted horizontally through direct contact or aerosol droplets. Vertical transmission has been confirmed only in chickens and turkeys. *M. gallisepticum* can only survive for short periods of time in substrates, but transmission from litter or feathers has been proposed from poultry houses to wild birds that visit them (52). Mycoplasmosis was documented to cause population effects in a density-dependent manner (37). *Mycoplasma synoviae* causes both respiratory and synovitis in domestic chickens. Fewer details exist on *M. synoviae* infection in wild birds, but antibody evidence of infection has been found in turkeys, house sparrows, and crows (27, 29, 79). Mycoplasmas have long been recognized for their tendency to interact with other pathogens. For example, the interaction between *M. gallisepticum*, respiratory viruses, and *Escherichia coli* is known as chronic respiratory disease (CRD) and interactions with Newcastle disease virus (NDV) or infectious bronchitis virus (IBV) appear to cause a synergistic effect with *M. gallisepticum* (43).

Infectious Bronchitis Virus (IBV) causes an acute, highly contagious respiratory disease occurring in chickens of all ages. Seropositivity against IBV has been reported in

wild birds such as pigeons (8). Jimenez *et. al.* reported on the widespread distribution of IBV in Costa Rican backyard chickens, and the prevalence of IBV antibodies in free-ranging Columbiformes (41). Species in the Orders Galliformes and Columbiformes would be considered at highest risk; however, Coronaviruses, genetically similar to IBV, are being increasingly detected in other avian species. IBV appears to have a wider host range than was previously thought, with recent isolations in a duck (50).

Hypothesis #2: Exposure to vector-borne diseases, such as *Hemoproteus* spp., will be higher in birds living in shade-grown coffee plantations, as a result of deforestation-related changes to vector population biology.

Background: Blood parasites have been reported from free roaming chickens in other tropical regions, and in wild birds, specifically in Costa Rica (71, 89, 97). The most common species reported in wild birds are *Hemoproteus sp*., *Leukocytozoon sp*. and *Plasmodium sp*. Specifically *Hemoproteus* sp. is a protozoan parasite that infects red blood cells of wild birds of many species, particularly waterfowl, raptors and passerines. Avian malaria, caused by *Plasmodium* has a worldwide distribution and is of great economic significance to the poultry industry. Organisms such as *P. gallinaceum, P. juxtanucleare and P. durae* may cause up to 90% mortality in poultry. Whereas *Plasmodium* is transmitted by mosquitoes, *Hemoproteus* is transmitted by *Culicoides* sp. or midges, and hippoboscid flies, and *Leucocytozoon* is transmitted by Simulidae black flies. Host susceptibility to infection by blood protozoans is poorly understood, as differences in prevalence among hosts can be associated with habitat preference and exposure to vectors (7). Infected birds are often weak, depressed, dyspneic and anorexic, although there is controversy about the clinical importance of hemoparasites for wild

birds. It appears that young birds are more susceptible than adults, and the most serious mortality generally occurs within the first few weeks of hatching. In the evolutionary ecology literature, hemoparasites have long been associated with sexual selection, and they have been assumed to represent a cost of reproduction and play a crucial role in the evolution of avian life histories (1, 58).

A recent experiment which duplicated the theory and methodology of the well-known Red Grouse experiments by Hudson et al., demonstrated detrimental effects on reproduction and parental condition in Blue Tits (39, 56). Through manipulation with antihelminthic treatment, Hudson was the first to demonstrated the regulating effects of *Trichostrongylus* on Red Grouse populations. Similarly, Merino utilized anti-hematozoan treatments to decrease *Hemoproteus* infection intensity and *Leucocytozoon* prevalence, showing that after manipulation, reproductive success and body condition of parents improved when compared with un-treated control groups. This provides evidence of the sub-lethal effects blood parasites have on bird populations.

Nonetheless, population effects due to infections with blood protozoans have been best characterized in penguins, Hawaiian endemic species and Blue Tits (6). The presence and prevalence of hemoparasite infection may be associated with season if the vector biology dictates activity, feeding and thus transmission, during certain times of the year (6). Theoretically, as is the case with *Plasmodium* in Hawaii, habitats which support higher densities of vectors are reflected in the prevalence of hemoparasites in birds.

Hypothesis #3: Prevalence and diversity of endo- and ecto-parasites will be higher in birds living in coffee-plantations due to artificial aggregation or contact with *Gallus domesticus*.

Background: Free-ranging wild birds are afflicted with numerous other parasites that occasionally cause illness and death. Traditionally, most parasitic infections were thought to be sub-clinical at the individual level and to cause negligible population effects. It is only recently that experiments with trichostrongyls, in natural populations of grouse in their native habitat in the United Kingdom demonstrates the impact that a parasite can have on the population dynamics of the bird host (39). There appears to be some evidence that the effect of parasitism on juvenile survival is particularly strong, when compared to temperate counterparts because parasite density is higher and is less affected by seasonal shifts (59).

Additionally, the decline of environmental quality has led to concerns about an increase in parasite prevalence in wildlife, such as aquatic birds (for example, (61)). Some of the internal parasites expected in the wild birds in this region of Costa Rica include cestodes, nematodes and protozoa. The transmission mechanism for these endoparasites varies, such that some cestodes require intermediate hosts, but birds become infected with nematodes and protozoa upon ingestion of infective stages found in the environment. Endoparasites are very common in wild birds, but they seldom cause death. Heavy burdens may reduce the vigor of the bird and serve as a predisposing factor for other disease agents, or the parasites may occlude the intestine or cause life-threatening malnutrition (16).

In addition to being vectors that transmit pathogens to birds, ectoparasites can directly cause morbidity and mortality. For example, even small numbers of adult ticks feeding on a small bird can cause anemia, reduced growth, weight loss, and contribute in other ways to a depressed state of health. Heavy infestations of lice, mites, fleas, flies,

and other biting insects have also been responsible for causing illness and even death of wild birds, especially among nestlings, and can translate to population effects (9, 16, 84). Clinical signs range from feather loss, and dermatitis to anemia. Mites of the genus *Knemidocoptes* are the primary cause of "mange" in domestic birds, and *Knemidocoptes spp* are commonly found in chickens (16). Lastly, ectoparasite load can be utilized as an indirect measure of host immune function, such that when a host is healthy, the level of parasite load or its effects may indicate the quality of its immune system (11, 59). We would expect birds living in shade-grown coffee to have a higher diversity of endoparsites, and a higher load of ectoparasites than those inhabiting forest fragments.

Hypothesis #4: Subjective measures of avian body condition will be higher in birds dwelling in forest fragments when compared to those living in coffee plantations.

Background: The assumption is that individuals in prime condition are better able to develop enhanced immune systems to ward off possible diseases and this can be indirectly quantified by measuring body condition (59, 60).Shade-grown coffee plantations present food subsidies for wild birds in the form of feeders for chickens, planted crops (e.g. corn, oranges, bananas, vegetables, etc), and in supporting a healthy, diverse insect population. On the other hand, birds inhabiting shade-grown coffee might experience fitness trade-offs due to the cost of immune defense against a higher prevalence or diversity of pathogens.

Hypothesis #5: *Enterobacteriaceae* spp. isolated from fecal samples of birds living in coffee-plantations will be similar in phenotypic and genotypic antimicrobial resistance profile to that of *Gallus domesticus* sharing their habitat and display a higher Antimicrobial Resistance (AMR) than isolates from forest birds.

Background: Antimicrobial Resistance is typically measured in commercially-reared animals and in the context of selection due to antibiotic use. However, I aimed to utilized the antimicrobial resistance profile (AMRP) as a model to study microbial distribution patterns and transmission in this habitat. Free range chickens could serve as reservoirs of antimicrobial resistance bacteria, which could be disseminated to birds utilizing shade coffee plantations. Current reports in the literature describe higher prevalence of antimicrobial resistance in isolates from wild birds associated with human-disturbed habitats, than of birds that are not exposed to human-associated activities. For example, *E. coli* isolates from black-headed gulls (*Larus ridibundus*) nesting in agricultural regions of the Czech republic displayed a 19% resistance to tetracycline, whereas only 7.6% of *E. coli* isolates from rooks (*Corvus frugilegus*) nesting in remote regions were resistant to tetracycline (24, 48). A recent study of the antimicrobial resistance of *E. coli* isolated from wild birds in the Artic reported low prevalence (8%), but proposes migratory birds as vehicles for transport of resistance genes (92).

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CHAPTER 2

INTRODUCTION TO DISSERTATION RESEARCH HYPOTHESES AND METHODOLOGY

Project Location

This research took place in the San Luis valley, located in the Northwest region of Costa Rica approximately 7 km from the town of Santa Elena in the Monteverde region, in the province of Guanacaste. This valley is on the Pacific, leeward side of the volcanic Cordillera de Tilarán mountain range. In this document, I separate the San Luis valley from the whole of the Monteverde region. In some texts, the Monteverde zone or region is described to include San Luis. This separation is justified given the fine scale of this research, the unique topographic characteristics of this area, and the differences in avian communities within just a 4 km hike (in turn, a reflection of the differences in weather and vegetation). This separation achieves 3 things: 1) highlights the lack of information outside of the immediate Monteverde region, 2) emphasizes that extrapolation of avian research from the Monteverde region to San Luis is not always appropriate, 3) stresses that attention to conservation issues outside of the "utopia" of the Monteverde region are needed. This research took place on the farms and forest lands above the town of San Luis, which stretches from approximately 900-1100 m of altitude. The San Luis region is considered tropical pre-montane cloud forest, with its vegetation and life forms defined by the high moisture and winds. The research described herein took place in the upper limits of Upper San Luis (San Luis Alto) at 1100 m. The culture, economy, geography,

weather and conservation of San Luis are inevitably tied to the rest of Monteverde and thus some background is indispensable.

History of the Monteverde Region--The Monteverde region, atop the Cordillera de Tilaran at 1440 m, was settled in the late 1940's by Quakers originating from the United States and escaping religious prosecution. Quaker organizations and their descendants continue to influence the infrastructure, organization and development of the region. The region collectively called "Monteverde" is comprised of the smaller towns of Cerro Plano and Monteverde (largely farms with approximately 700 inhabitants) and the much larger town of Santa Elena (7,000 inhabitants). Santa Elena is the commercial, transportation and tourism hub of the region. It receives approximately 200,000 visitors a year, mostly seeking its natural beauty in the three large private reserves (the Monteverde Cloud Forest, the Children's Eternal Rainforest and the Santa Elena Reserve), which together make up more than 17,000 hectares. Home of the now extinct golden toad and the elusive Resplendent Quetzal, Monteverde's forests have been a focus of scientific research for decades (REFS). Historically, the Monteverde region was completely covered in closed canopy forest and all non-forested areas existing currently are due to human activity—previously slash and burning and more recently, cattle grazing.

The Physical Environment of Monteverde and San Luis—The Monteverde region, positioned perpendicular to the northeasterly wind trade flow, with its unique geology and rugged mountainous terrain provides a distinctive physical environment that promotes rich biodiversity. The upper ridges of the Cordillera de Tilaran are likely

volcanic in nature dating to the Quartenary age (4). The mountain ridges have been shaped over time by erosion and the numerous rivers and tributaries of the region. Major streams rapidly descend both slopes of the Cordillera often forming spectacular waterfalls and rapids. These streams and their tributaries carve deep gorges, forming steep ridges. The San Luis valley is defined by narrow catchments separated by ridges with steep flanks, whereby frequent landslides, triggered by torrential rains and earthquakes, have dampened the topography.

The hydrology of Monteverde is primarily influenced by precipitation and cloud water. Precipitations records are available and will be discussed later, but cloud water measurements are often overlooked. The Monteverde region consists primarily of primary and secondary cloud forests, farms and livestock pasture. The forests of Monteverde are considered tropical montane cloud forests (TMCF) and are a product of frequent cloud cover throughout the year, low levels of solar radiation, and high precipitation. Consequently, there is a very high regional plant biodiversity squeezed into small regions in “bands” parallel to the Cordillera, and arranged according to the six Holdridge life zones (8). According to these life zones, San Luis is considered premontane wet forest (PMWF). Located at 900-1200 m, San Luis receives an annual rainfall of 2000-2500 mm compared to 2500-4000 mm recorded for areas near the Monteverde Cloud Forest Reserve. Upper San Luis, as opposed to the rest of the Pacific Slope, benefits from some wind-blown mist during the 5-6 month long dry season; however, it is much less than in the cloud forests, where, due to frequent mist and cloud cover during the dry season, remain wet all year long (8).

There are currently 3021 vascular plants described in the Monteverde region, of which 755 are species of trees, although records are continuously being added. The trees in the Monteverde region are shorter (10-20 m), denser and laden with mosses, epiphytes and trunk climbers. However, San Luis, being on the Pacific slope, lies on the leeward side of the tradewinds and thus trees tend to be taller (25-40 m), contained in a seasonal forest that is mostly evergreen (less than 10% of the canopy leafless during the dry season) and has moderate epiphyte diversity and abundance. Table 2.1 illustrates a list of characteristic trees of the Pacific Montane Wet Forest (PMWF). Of particular importance to my research are those species which offer a critical resource to the resident and migrant avifauna (Table 2.1). Several invasive species have recently invaded Monteverde, either transported by humans as ornamentals (*Impatients walleriana*), through transport by the relative explosion of horses for tourism, or by species of birds that appear to be moving upward on the slope presumably due to recent warming conditions (Hernandez-Divers, personal observation; A. Pounds pers. Communication, REF). Unlike the Monteverde region, where the dense understory is populated by shrubs, treelets, fern trees and herbs, the understory of the PMW forest is fairly open with few shrubs and tree saplings. The San Luis valley has rich soils, supporting the only coffee production in the region.

Weather in Monteverde and San Luis--The Monteverde region is defined by its weather conditions. Most of the data collected in this region comes from the Monteverde Institute, located at 1420 m elevation. Topographic position and exposure to trade winds and trade-wind driven clouds and rain shape a variety of microclimates in Monteverde

(4). Mean annual precipitation in Monteverde is estimated at 2.5 m. However, precipitation measurements underestimate moisture from wind-driven clouds. Three distinct weather seasons are defined in Monteverde: 1) wet season (May-October); whereby mornings are clear, yet cloud accumulation throughout the day results in rain in the afternoon and evening, 2) the transitional season (November-January) which brings very strong trade winds, clouds and wind-driven rain, and 3) dry season (February-April) characterized by moderate trade winds, clear skies and wind-driven mist, particularly at night and early morning. Weather in Monteverde is influenced by the migration of the Intertropical Convergence Zone (ITCZ), and three weather systems: 1) polar cold fronts, 2) Pacific fronts and 3) hurricanes. The ITCZ is an area of low pressure that forms where the Northeast Trade Winds meet the Southeast Trade Winds near the earth's equator. As these winds converge, moist air is forced upward. This causes water vapor to condense, or be "squeezed" out, as the air cools and rises, resulting in a band of heavy precipitation around the globe The ITCZ is directly above Costa Rica during the wet season, characterized by intense convective precipitation events. During the transitional and dry seasons, the ITCZ is located south of Costa Rica, and moisture is brought to the region via northeasterly trade winds from the Caribbean Sea (4). Polar fronts are a result of cold, dry polar air occurring frequently from December to February. As they sweep over the Gulf of Mexico and the Caribbean Sea, warm, moist air ascends above cooler, denser air. With adiabatic cooling, clouds form and the trade winds push these clouds and air masses over the Tilaran Cordillera. Orographic uplift and adiabatic cooling translates to intense wind-driven precipitation and mist in Monteverde. Pacific fronts resulting from tropical low-pressure systems in the Caribbean; occur from August to October, also

corresponding to the hurricane season. By reversing surface winds, these fronts draw moist air from the Pacific Ocean over Monteverde. Again, orographic uplift and adiabatic cooling produce clouds that produce cloud immersion and rain. Hurricanes are relatively rare in this region, but rainfall from September-October reflects effects of tropical depressions. Although the general weather trends also apply to San Luis, there are some specifics worth mentioning. Being on the Pacific slope, San Luis experiences longer dry seasons (20). San Luis is severely buffeted by the easterly trade winds during January-March with average wind speeds of 20 m/s (44.7 mph; UGA San Luis Research Station, 2007). Trade winds hit the Cordillera at a right angle, creating a moisture shadow on the Pacific slope, which means that life zones occur in narrower bands on the Pacific slope than on the Caribbean slope. Figs 2.1 a & b show the monthly averages of precipitation and wind speed for San Luis for my research period (June 2005-January 2008).

Economics and Conservation of San Luis--Whereas Monteverde has enjoyed international attention from the scientific community, until recently, none of that focus had spilled into San Luis. This is largely due to the geographic isolation of the valley. Until very recently, direct access from San Luis to the other towns depended on a gravel road that climbed a steep hillside, frequently prone to landslides and unreliable during the rainy season. A section of that road was manually paved by the San Luis community five years ago, allowing regular trade, employment opportunities, and more regular tourist visitation to and from Santa Elena. Considering the conservation themes that permeate my project, it is important to understand the socioecomomic and cultural forces that are prevalent in this region. The San Luis Valley is a mosaic of agricultural land (pasture for

livestock, monoculture crops, polyculture crops, small coffee parcels, and both primary and secondary forest fragments). The town of San Luis is comprised of approximately 400 inhabitants, and the residences are concentrated into two sections termed "Upper San Luis" and "Lower San Luis". There is pre-colonial evidence of human inhabitation in the region, although it is thought to have been sparse. The first Europeans arrived in the valley in the 1920's as subsistence farmers. It was not until the 1960's, however, that the town's progress was thrust forward with the construction of a road system, two elementary schools, and the construction of the cheese factory in Santa Elena, as well as the nation-wide promotion of coffee production.

The people of San Luis are conservative, strongly rooted in family values and the Christian religion. The San Luis Comprehensive Development Association, a committee of community members, is the most prevalent form of organization. The principal limitation of this town is the lack of post-elementary education. Students must travel to Santa Elena to receive 6^{th}-12^{th} education, a trip that involves 1.5 hrs of walking each way, and which leads to a high level of attrition. Approximately 8% of the population has graduated from high school, and only about 3% have received any college training. According to Noe Vargas, previous President of the Development Association,

"*…The common vision for the San Luis community is one that battles for a better quality of life, within the framework of sustainability…. the community is always trying to find economic alternatives, while taking into account the appropriate management of ecotourism, retaining our cultural values, and promoting environmental conservation.*"

San Luis is a rural community with its primary sources of income as dairy cattle, coffee and tourism. Milk is marketed through the Producers of Monteverde Company, the

same organization that runs the Santa Elena Cheese factory and which produces cheese, ice cream and other dairy products both for national consumption and export. Many families are tied to the tourism industry either through direct employment in tourism facilities (including the UGA San Luis Research Station), or through sale of their crops and crafts to these facilities. Coffee has been the traditional cash crop in San Luis. In the 1960's, a Coffee Processing plant was built and marketing was established. After 1986, the plant changed hands to a cooperative of producers from the area, who grow, process and sell the coffee with the Café Monteverde brand.

The UGA San Luis Research Station, which was utilized as the base for this project, is located on Upper San Luis. The Station consists of 66 hectares of secondary and primary growth forest and includes the riparian habitat of the San Luis River and two smaller creeks, La Bruja and La Londra. The property stretches from the boundaries of Upper San Luis to the borders of the Monteverde Cloud Forest Reserve and the Children's Eternal Rainforest. UGA San Luis Research Station is a model of sustainable ecotourism and it is dedicated to protecting the biological and social environment of the area.

Avifauna and Conservation in Monteverde--Given the spectacular coloration of several species, interesting behaviors, ease of observation of a majority of species (because of shorter canopies and a high number of ground-dwellers) and abundance, birds have been a major focus of scientific research in the Monteverde region. Monteverde, straddling the Continental Divide, encompasses the "wet" Pacific slope avifauna, the "wet" Caribbean slope avifauna, and the highland fauna. In addition, South

and North American migrants are added to this mix, creating an impressive diversity. As mentioned, the Holridge life zones occur in bands that parallel the Tilaran Cordillera ridge and thus habitat specialization for birds is compressed into a small spatial scale.

The habitat changes dramatically in the Monteverde region with small changes in altitude, such that six life zones occur within a 600 m elevational range (8). Avian community composition often changes rapidly with elevation in the Neotropics and specifically in Costa Rica, bird species composition changes substantially from 500-1000 m, with bird specie richness declining dramatically above 1500 m (2, 21). In the Monteverde area there is evidence of both high bird species turnover across elevations and declining bird species richness with elevation (20, 21).

This has important implications for research. For one thing, extrapolations cannot be made within relatively short distances due to significant differences of avian community composition. Thus, comparative studies dictate that field sites are located close to one another. Additionally, a large number of studies have focused on species found specifically in the Monteverde reserves (e.g. Resplendent quetzals, brown jays, black-faced solitaires, collared red-starts, etc) and on features which characterize the avifauna in the Monteverde region (e.g. mixed species flock, foraging and social behavior, altitudinal migration, etc) (11-13, 17) . Yet, relatively little data has been collected on the avifauna outside the protected zones. Lastly, much of the research has focused on single-species and has ignored important community questions, which, in turn, is tied to important conservation themes.

Project Methodology

Field Sites--The UGA San Luis Research Station (10 16'57,117" N 84 47'53,747" W) served as the base for all laboratory processing. Six field sites were chosen and are described herein. Although all sites are considered to be embedded within the classification of the pre-montane lifezone, the geographic location of each site and the extreme weather in San Luis translates to subtle changes in climactic conditions and vegetation.

Finca La Bella: The Primary Research Site

In the 1980's, Anne Kriebel, a Quaker living in Santa Elena was working in San Luis to improve education, health and nutrition of its inhabitants. Anne died suddenly, and in her honor, the Quaker Earthcare Witness organization raised funds to purchase 122 acres, which became Finca La Bella. Finca La Bella (literally "The Beautiful Farm") is a cooperative farm that has provided 25-yr renewable leases of 1-2 hectare parcels to approximately 24 families. The Farm sits in the center of Upper San Luis. Anne Kriebel believed in sustainable agricultural practices, and thus, the policy of the Farm is to protect the forest fragments within it, and to promote agricultural alternatives, such as shade-grown coffee, that benefit both the farmers and the environment. Whereas in the 1960's-70's most of the coffee grown in the San Luis valley was sun-grown, coffee parcels have been converted to shade-grown through replanting. Currently the farm is managed by a committee of "parceleros".

Four sites were located within Finca La Bella and two within the UGA San Luis Research Station grounds. The sites, shade-grown coffee parcels, were named as follows:

Joel, Alvaro, and Gilberth. Initially, another site, Vargas, was chosen, but due to a change in ownership, the Joel site was chosen to replace Vargas. The forest fragments are named: Los Nenes, Zapote and Peña. The three shade-grown coffee parcels were chosen because of all the parcels in the region they cultivated the most coffee under "shade-grown" conditions, they appeared to have a significant avian community, they were geographically separated from one another, and finally, the owners were amenable to research conducted on their property. The forest sites were chosen to be old secondary growth (~75 yrs), were separate from one another, were sites considered "embedded" within larger forest tracks and finally were relatively accessible.

"Gilberth"

This parcel belongs to Gilberth Lobo Navarro, is inhabited by his family (5 members), lies at approximately 1143 m elevation (N 10° 17.140' W 84° 82.204) and is 1.5 hectares in size. Its boundaries are the main internal dirt road (two sides) in Finca La Bella, an adjacent parcel and a forest fragment. Across from the road on one side are other shade-grown coffee parcels, and on the other, a cattle pasture. It can be described as a multiculture crop system and its heterogeneity and crop rotation from year to year, make it difficult to describe. The parcel is hilly and an irrigation creek courses through the middle of it. It is composed 75% of rows of coffee, which are interspersed in between native and fruiting trees. Other crops include sugar cane, corn, herbs, and a variety of vegetables for household consumption. The parcel, as is typical for all parcels in the region, is surrounded by windbreak trees, and subdivided into smaller sections by rows (approximately 4 m wide) of windbreak trees, making the parcel appear to have 7 distinct "rooms" within it (Figure 2.2). The majority of trees are within windbreaks lines.

However, other mature trees are distributed throughout in a random manner. The family has a flock of free-roaming chickens (~20 animals), as well as a flock of 10 "broilers" that are always contained in a chicken coop. Figures 2.3a & b.

"Alvaro"

This parcel belongs to Alvaro Vega Anchía, is inhabited by his family (3 members), lies at 1132 m of elevation (N 10° 17.181' W 84° 48.368') and is approximately 2.5 hectares, although only 1.5 hectares are planted with coffee--the rest is a large garden he uses for tourist groups and a slice of forest. The parcel is on the edge of a steep slope, overlooking the Southern portion of Finca La Bella and is therefore more exposed to wind than Gilberth. It is bounded by another parcel, a forest fragment, and an extremely steep, rocky, grassy, uncultivated ridge on two sides. One of those sides forms the "canyon" of the El Socorro creek, the primary water source for Finca La Bella. In comparison to Gilberth, Alvaro does not contain as many large native trees within the parcel, but has been heavily replanted with fruiting trees. The windbreak trees surround 2 of the 4 sizes of the parcel, and further subdivide the parcel into 3 major areas. Due to its location on the side of a slope, and the relative scarcity of larger native trees, Alvaro's parcel is drier and sunnier than Gilberth's. Alvaro does not own chickens, but his immediate neighbor has a large flock (~35) which freely roams into sections of Alvaro's property. Figures 2.4a&b.

"Joel"

This parcel, 2 ha in size, lies at 1073 m (N 10° 16.973' W 084° 48.347') belongs to Joel and is not inhabited, although Joel has recently built a small structure. Of the 2 has, only 1 is planted and the rest is a forest fragment that divides his parcel from an

adjacent farm. Of the planted region, 90% of the parcel contains coffee. The parcel is bordered by the San Luis road, his forest fragment, and two adjacent parcels. Joel has planted several fruiting trees (mango, banana, papaya, avocado) throughout the parcel. Windbreak tree rows divide the parcel into 3 sections. Other crops include beans, herbs, and garden vegetables. Joe's parcel most resembles Alvaro's in its tree density, yet it is much more protected from the wind than Alvaro's. Figures 2.5a &b.

"Zapote"

This site is embedded within a rectangular forest fragment (30 ha) that belongs to the UGA San Luis Research Station. Approximately 1.5 ha of a forest patch that included the Zapote trail was chosen for this site. This trail connects the UGA San Luis Research Station grounds to the closest farm, and lies at approximately 1158 m (N 10 17.051' W 84 47.946'). It is located on the top of a ridge, and is composed of old growth secondary forest. Zapote is the forest fragment closest to the UGA San Luis Research Station. Figures 2.6a & b illustrate Zapote.

"Peña"

The largest expanse of forest on the UGA San Luis Research Station includes the riparian habitat of the San Luis river. The **Peña** trail (and the 1.5 ha site) lies on a long ridge parallel to the river, and is composed of secondary forest. Figures 2.8a& b illustrate Peña.

"Los Nenes"

This 1.5 ha site is embedded within a protected patch of forest inside Finca La Bella and is the forest fragment in closest proximity to coffee plantations. It lies on the upward slope of the "Trocha" road that connects San Luis to Santa Elena, at 1158 m (N 10°

17.279’ W 84° 48.176’). It is composed of secondary forest, currently preserved for bird watching and tourism. Figures 2.7a & b illustrate Los Nenes.

Table 2.1 illustrates size, percent canopy cover, average tree height, tree width, tree density and the most common trees in each site.

Avian Capture--Birds were captured with mist nets following standard methodology (REF). Approximately 8-14 nylon mist nets (38 mm nylon, 4 panels, either 6 or 9 mm in length) were erected the evening before a capture day and maintained furled. The morning of capture, the nets were opened between 5-7 AM, and maintained open until 1-2 PM or until 10 birds not previously banded were captured per day. The nets were monitored every 30 min. Nets were opened on two alternating sites, to avoid capturing in one site on two consecutive days, which leads to a decrease in capture effort. Human activity was not permitted near the nets while they were open, other than to extract birds. The nets were closed early if it rained substantially, or if it was very windy. Wet nylon, in combination with wet feathers, inflicts abrasions and bruising small birds. Additionally, small birds become hypothermic in a short time when captured in a mist net during the rain. Wind renders the mist nets visible (by movement and by debris that becomes lodged on the nets), reducing capture effort. Birds were extracted from the nets and placed in disposable brown paper bags. Time of capture was recorded. Birds were transported to a “base camp” where they were processed. Birds were processed immediately to minimize holding time. In most cases, birds were released within 20 min of extraction from the nets, and released near the point of capture. If a bird suffered an injury that would render

it non-releasable, a rare event, it was immediately euthanized by cervical dislocation. All avian capture and handling techniques were reviewed and approved by the University of Georgia's Animal Care and Use Committee.

Bird Processing--A bird was weighed while still in the bag. Birds were banded on the left leg if utilizing the smallest of band size, and on the right leg if any other size was used. The bands were color coded for the site and consecutively numbered. They were made of ultra-violet sensitive plastic and are expected to degrade and fall off after 3 years. The following measurements were obtained: culmen, tibiotarsus, wing cord. If sexually dimorphic, sex was noted. If displaying juvenile characteristics, age was determined. The following subjective scores were obtained: body condition score based on pectoral muscle density (scored from 1-5/5; 1=emaciated, 2=thin, 3=ideal, 4=overweight, 5=obese; (18)) and ectoparasites score (scored from 1-5; 1=no mites noted, 2=1-25 mites, 3=25-50, 4=50-100, 5=>100). Other information noted and recorded included: molting pattern, any physical abnormality, and presence of brood patch or egg in the abdomen. Mites were collected by sharply dissecting a small window of mite and mite eggs on the wing feathers, which were stored in 70% ethanol. Body and feather lice, ticks or hippoboscid flies were collected directly. Feces deposited on paper bags were sampled aseptically with a culturette (Becton Dickinson BBL Culture Swab with Aimes media, BD, Franklin Lakes, NJ) for microbiology. If enough fecal material was available, additional sample was collected and stored in 2.5% potassium dichromate for endoparasite examination. Birds that weighed >20 g were restrained for jugular venipuncture. Birds weighing < 20 g were sampled via superficial ulnar vein puncture via capillary action with heparin-lined microhematocrit tubes. No more than 1% of body

weight in blood volume was collected. Upon blood collection, a blood smear was made immediately, dried and stored. The remaining blood was stored in plasma separator tubes that contained lithium heparin (Becton Dickinson 0.6 ml Microtainer green top tubes, Franklin Lakes, NJ). Focal avian species underwent additional testing. A choanal swab was obtain by vigorously rubbing a small, sterile, polyester tipped applicator (Pur-Wraps, Hardwood products, Guilford, Maine) in the choanal cleft. The swabs were immersed into 1.5 ml microcentrifuge tubes containing 300 μml of sterile PBS. A cloacal swab was obtained with small, sterile cotton swabs which were immersed in sterile microcentrifuge tubes containing Dulbecco's Modification of Eagle's Medium (DMEM; Mediatech, Manassas, VA) cell culture media with added antimicrobials (amphotercin B at 1%, gentamicin at 0.1% and penicillin/streptinomycin at a concentration of 5000 IUs/ml)(9). These swabs were frozen for later processing. All samples were maintained in an ice chest until further processing.

In-country Laboratory Processing--All samples were processed within 6-8 hrs of collection. Blood was centrifuged and both whole cells and plasma were immediately frozen. Blood smears were stained with Wright's stain, dried and stored. Fecal bacteria were propagated on MacConkey media plates by incubating plates at 37° C for 12-18 hrs. Individual colonies were aseptically collected, introduced into sterile stab storage media (tryptone 0.2 %, yeast extract 0.02 %, and agar 0.5 % in distilled water) and maintained at 4° C until export. Cloacal swabs were immediately frozen. DNA was extracted from choanal swabs every day, utilizing a commercial available Qiamp Mini kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. The resultant solution was frozen at -70° C until processing. Biological samples were imported into the USA

under a United States Department of Agriculture (USDA) permit and an export permit issued by the Ministry of Agriculture of Costa Rica. All biological samples were prepared for importation following USDA guidelines for pathogen inactivation.

Laboratory Methodology (USA)—

Hemoparasites. Blood smears were examined for the presence or absence of hemoparasites in a standard manner (19). Briefly, smears were examined at 10X for the presence of larger parasites, such as microfilaria, and to locate an adequate monocellular layer, and then at 100X under immersion oil for 10 min to locate all intracelluarl parasites. All blood smears were subsequently reviewed by a clinical pathologist who confirmed the results. At least five fresh fecal samples were collected from each flock. Fecal samples were preserved in a 2.5% potassium dichromate solution until examined. Samples were examined directly, by fecal smear, and by standard flotation technique with Sheather's sugar solution and examined microscopically (10, 19). Ectoparasites were collected and stored in 70% ethanol for later identification using morphologic characteristics by an avian veterinary parasitologist.

Serology. Blood tubes collected in the field were maintained in a cooler with ice and centrifuged no more than 8 hrs after collection. Following centrifugation, the serum was transferred to cryovials, and frozen in a -80° C freezer until processing. In order to determine disease seroprevalence, hemagglutination inhibition was performed for Newcastle Disease (NDV), Avian Infectious Bronchitis (IBV), and *Mycoplasma gallisepticum*, (MG) and *M. synoviae* (MS) at either the School of Veterinary Medicine,

Universidad Nacional de Costa Rica or at the University of Georgia Poultry Diagnostic and Research Center (PDRC), Athens (7).

***Mycoplasma spp.*nucleic acid detection.** Choanal swabs were collected from a limited number of animals to determine the presence of *Mycoplasma gallisepticum* and/or *M. synoviae* nucleic acid. The swabs were placed into 1.5 ml microcentrifuge tubes containing 300 μml of sterile PBS. The day of collection, DNA was extracted utilizing a commercial available Qiamp Mini kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. The resultant solution was frozen at -70° C until processing. To determine the presence of *Mycoplasma gallisepticum* DNA, a Real-time Taqman® polymerase chain reaction (R-PCR) was utilized as previously described (Callison). A plasmid containing the amplification target sequence of the MGA0319 gene (lp gene; GenBank Accession # NC_004829) previously described by Callison *et. al*., was utilized as positive control (3). To determine the presence of *Mycoplasma synoviae* DNA in each sample, a PCR assay was utilized as previously described by Lauerman *et. al* (10). Positive controls utilized were reference strains WVU1853 and F102AS.

Paramyxovirus amplification and isolation. Cloacal swabs were aseptically collected from selected animals from three flocks. The swabs were immersed in sterile microcentrifuge tubes containing Dulbecco's Modification of Eagle's Medium (DMEM; Mediatech, Manassas, VA) cell culture media with added antimicrobials (amphotercin B at 1%, gentamicin at 0.1% and penicillin/streptinomycin at a concentration of 5000 IUs/ml) and frozen for later processing. To realize virus amplification and isolation, the tubes were vortexed, the swabs discarded, and the tubes centrifuged at 10,000 rpm for 5 min. The supernatant was loaded on to a sterile syringe and injected into ten day old

embryonated chicken eggs. After 72 hrs, allantoic fluid was collected and utilized for a hemagglutination assay. One drop of fluid from each isolate was placed on cards designed to inactivate cells, yet capture and preserve nucleic acid, (FTA© classic cards, Whatman International Ltd, Springfield Mill, James Whatman Way, Maidstone, Kent, UK) for storage. Extraction of DNA from the FTA cards for the molecular detection of Newcastle disease virus was performed as previously described (16).

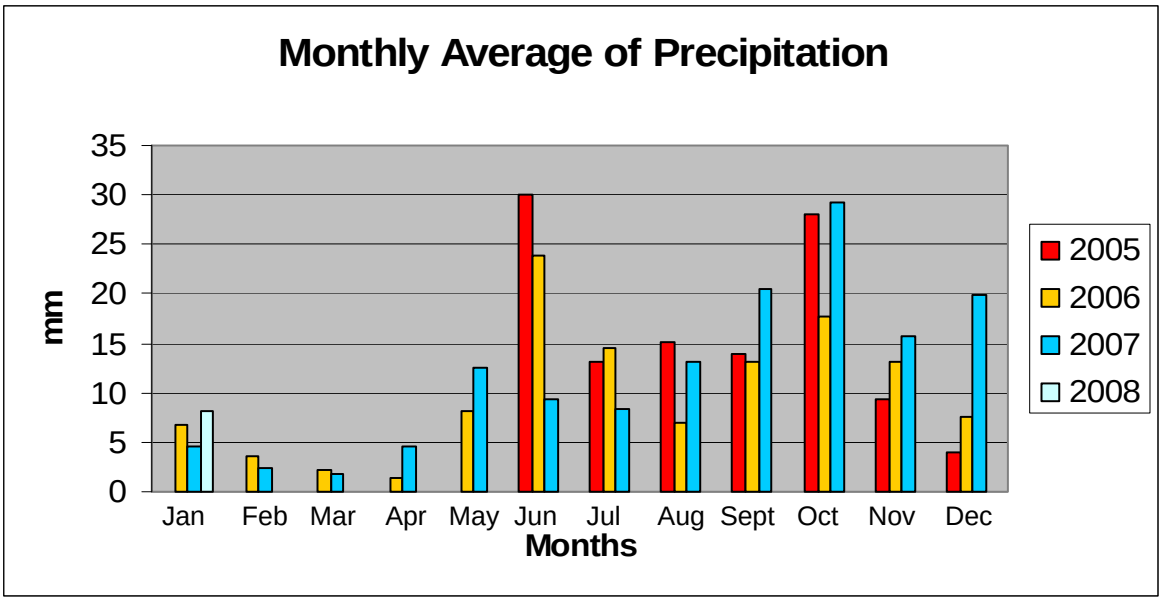
Phenotypic Antimicrobial resistance patterns of fecal flora. Fresh fecal samples were collected from the center of an excrement mound, taking care not to touch anything else, with culturette swabs (Becton Dickinson BBL Culture Swab with aimes media, BD, Franklin Lakes, NJ), maintained in an ice chest with frozen gel packs and transported back to a laboratory for processing within 8 hrs of collection. Fecal bacteria was propagated on MacConkey media plates, incubated at 37° C for 12-18 hrs. Individual colonies were aseptically collected, introduced into stab storage media (tryptone 0.2 %, yeast extract 0.02 %, and agar 0.5 % in distilled water) and maintained at 4° C until export. Once in the USA, bacteria was streaked for isolation on to both blood and MacConkey agar plates and incubated at 35±2° C for 12-18 hrs to confirm purity. Bacterial isolates were then stored in freezer media (1% peptone, 15% glycerol in distilled water), and frozen at -80° C until further processing. Bacterial identification was done through standard biochemical reactions (triple sugar iron, motility, indole, ornithine, oxidase, and citrate) or with commercially available *Enterobacteriacae* identification strips (API 20E; bioMerieux USA, Durham, NC). Minimum inhibitory concentrations (MIC) were determined using TREK, diagnostic system plates following the manufacturer's instructions and previously described standards (5). MIC plates

contained a series of titrations of the following antibiotics: amikacin, tetracycline, ticarcillin, ampicillin, amoxicillin with clauvulinic acid, clindamycin, chloramphenicol, enrofloxacin, erythromycin, difloxicin, gentamicin, imipenem, orbifloxicin, marbofloxicin, trimethoprim sulfa, cefazolin, cefoxitin, cefpodoxime, ceftiofur, cephalothin, amikacin, spectinomycin, penicillin, oxacillin, rifampin and tilmicosin (Trek Diagnostics, Cleveland, OH). The plates were incubated at 37° C for 12 hrs, at which time they were visually inspected for growth. The plate cells which displayed growth were recorded and the first cell without growth was deemed the MIC endpoint. Resistance breakpoints were determined based on previously published data (14).

Genotypic Antimicrobial resistance patterns of fecal flora. To determine the presence of Class I Integron, t*et(A)* and *tet(B)*, whole cell templates were made in a standard manner from pure culture stock. Briefly, isolates were plated on to blood agar and after 7 hrs of incubation at 37º C, all growth was collected and incubated in 2 ml of BHI broth for 12 hours at 37ºC while stirring. Each tube was vortexed and 1.5 ml of the culture was transferred to a microcentrifuge tube where it was centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 1 ml of 100% ethanol and vortexed again thoroughly. The tube was allowed to incubate at room temperature for 10 min and again centrifuged at 7000 rpm for 10 min. Once again the supernatant was discarded and the pellet resuspended in 1 ml of 1X PBS and vortexed thoroughly. It was incubated again at room temperature for 10 min and centrifuged at 7000 rpm for 10 min. The PBS was decanted and the pellet suspended in 1 ml of sterile, deionized water and vortexed thoroughly, incubating it at room temperature for 10 min prior to freezing in -20° C for long term storage until further processing. Polymerase chain reaction was used

to identify the samples that contained drug resistance genes such as Class I Integron (*int1*), and t*et(A)* and *tet(B)* as previously described (1, 6, 15). A list of primers utilized is summarized on Table 1. The PCR positive control used was *Salmonella enterica serotype Typhimurium* DT104 and the negative control was sterile, DNA-free water.

Figure 2.1 a & b. Monthly averages of daily precipitation (mm) and wind speed (kmph) for research periods June 2005-December 2008.



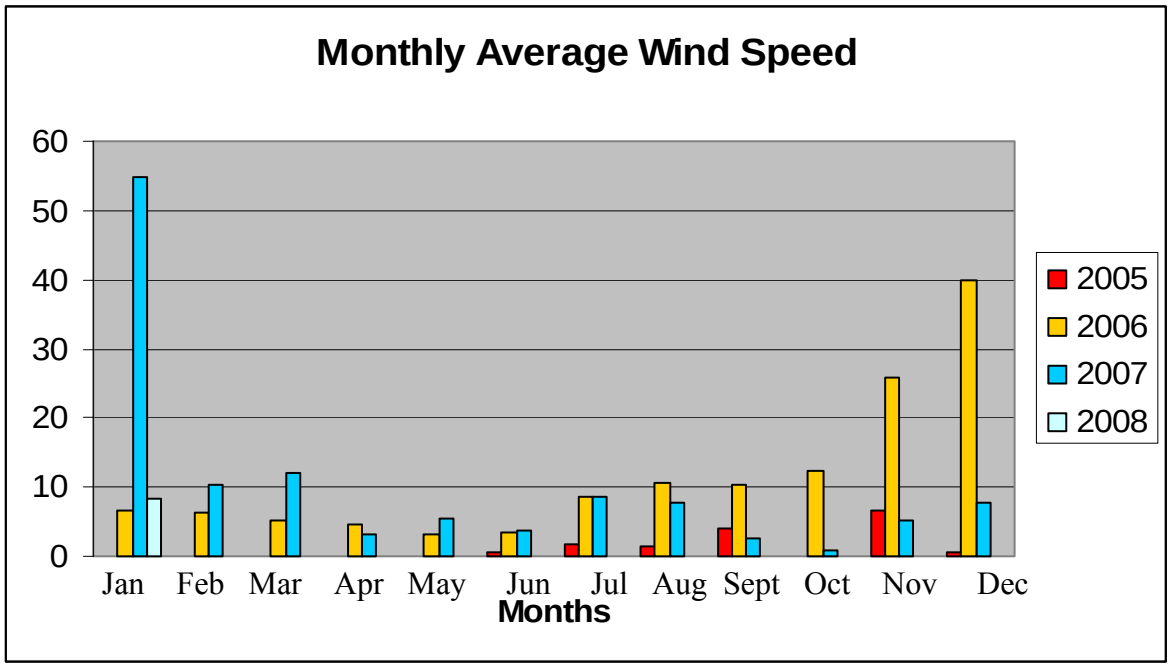


Figure 2.2 This figure illustrates, to scale, Gilberth Lobo's parcel. It is meant to show the heterogeneity of the shade-grown coffee plantations, such that many crops are planted around coffee plants; lines of windbreak trees subdivide these parcels into smaller subsections; and

Figures 2.3a & b illustrate Gilberth Lobo's parcel. Note the coffee plants growing within native trees.

Figure 2.4a illustrates the view from one of the borders of Alvaro's parcel, showing the bluff that exposes this parcel to wind, and looking at other parcels on the southern portion of Finca La Bella.. Figure 2.4b shows coffee plants growing under banana trees, native trees, and a windbreak tree line in the background during a misty morning.

Figure 2.5a illustrates Joel's parcel, with coffee at different stages of growth, banana trees, a line of windbreak trees, and the characteristic mist that blankets San Luis; 2.5b shows that Joel's parcel had the lowest overall canopy cover.

Figure 2.6. The Zapote trail, as shown here, runs through the middle of the Zapote site. Both photographs illustrate mist nets placed on the Zapote site.

Figure 2.7a & b illustrating mist nets placed at Los Nenes.

Figure 2.8 illustrating the Peña forest.

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CHAPTER 3

A SURVEY OF AVIAN PATHOGENS OF BACKYARD POULTRY IN NORTHWESTERN ECUADOR

INTRODUCTION

Infectious diseases have long been recognized as a major factor affecting the population dynamics of free-ranging species (3). More recently, infectious diseases have been responsible for major declines of wild animal populations (44). Therefore, conservation efforts should consider the health issues that affect wildlife (10, 23) . In the past, studies of diseases in free-ranging wildlife were limited to zoonoses or to species or diseases of economic significance or were triggered by an event that caused a significant negative impact, for example the epizootic of morbilli viruses in marine mammals (42). Disease issues affecting wildlife have been linked to a variety of anthropogenic activities,(9) such as introducing new pathogens or exotic species or modifying habitat, leading to an increase in disease prevalence or environmental contamination (17, 28, 33). However, a new approach should be pursued that encourages the study of human effects on the health of populations, communities, or ecosystems before catastrophic events occur (2, 9, 10).

Ecuador is recognized as a high priority area for biodiversity conservation (7, 27, 36). Within Ecuador, the Choco-Andean region shelters 4 % of bird species on the planet. Given that it has the highest number of endemic bird species, this area is considered by scientists as a "global priority zone" (36). Therefore, preserving avian biodiversity in this region should be a priority (32). As evidenced by disease issues in the conservation of Hawaiian birds and recent emerging diseases such as West Nile virus and *Mycoplasma* species conjunctivitis, infectious disease can play a significant role in the dynamics of wild avian populations and should be investigated (12, 31, 41). Unfortunately, few studies shed light on disease agents that affect neotropical wild birds (13, 15).

The project we describe is part of a larger ecologic study aimed to understand the relationship between human-induced land use changes and the prevalence and diversity of pathogens in avian communities in 4 land use types: primary and secondary low montane forest within a private reserve, “eco-friendly” agricultural land, and active agricultural land. Although the effect of anthropogenic habitat modification on birds has received substantial attention, studies have focused on the context of population distribution, diversity, and abundance (5, 6, 11, 19, 20). None of these studies included a component that investigated health or disease.

The first phase of this project involved describing selected pathogens of a variety of free-roaming, backyard chickens in northwestern Ecuador (lat S 0˚ 13’ 00”;long W 78˚ 63’ 30”) immediately surrounding the Maquipucuna Reserve. Chickens are often used as sentinel species to monitor the presence and prevalence of certain infectious diseases and in most cases are considered good indicators of avian disease agents in a given environment (21, 28, 32). In this study, chickens were used to determine the pathogens to

which wild birds that exploit anthropogenic habitats might be exposed. Secondly, backyard flocks of chickens living in and near the village often stray into forest patches and share food and water sources with wild birds, potentially creating opportunity for disease transmission to free-ranging birds. Examples of avian species that were observed within anthropogenic habitats and either feed, roost, or both within villages and farms include the smooth-billed ani (*Crotophaga ani),* common nighthawk *(Chordeiles minor),*white-collared swift *(Streptoprocne zonaris),* Pacific hornero *(Furnarius cinnamomeus),* lemon-rumped tanager *(Ramphocelus icteronotus),* swallow tanager (*Tersina viridis),* blue and white swallow *(Notiochelidon cyanoleuca cyanoleuca),* blue-black grassquit (*Volatinia jacarina),* shiny cowbird (*Molothrus bonariensis aequatorialis*), turkey vulture (*Cathartes aura*), black vulture (*Coragyps atratus*), cattle egret *(Bubulcus ibis*), and a variety of members of the Tyrannidae family (flycatchers) and Troglodytidae (wrens). Birds that utilize forest into which chickens roam and that have ecologic behaviors that might put them at risk of coming into contact with poultry and poultry feces include ground birds (or those that spend a significant amount of time on the ground) such as Tynamidae (tinamous), Columbidae (pigeons and doves), Thamnophilidae (antbirds, antshrikes), Caprimulgidae (nightjars and nighthawks), Furnariidae (foliage gleaners and leaf tossers), Formicaridae (antpittas), Emberizidae (finches and grassquits) or Turdidae (thrushes); and birds that might either consume chickens or aggregate near foodstuff consumed by chickens such as Cathartidae (vultures), some members of Accipitridae and Falconidae (hawks, eagles and falcons), Cracidae (guans), and Odontophoridae (quails). This list is not complete, as birds that do not spend time on the ground, but roost, nest, or feed in trees where chickens are roosting

might also be considered. Lastly, “eco-friendly” agricultural land, such as shade-grown coffee plantations, is promoted in this region as a way to enhance biodiversity because it provides more suitable habitat and supplemental food (i.e,, banana) for forest birds than pasture, monoculture crops, or other types of traditional agriculture. However, shade-grown coffee plantations are managed much like traditional agricultural land, which often translates to domestic animals having access to these “forests”, creating an additional source of contact between chickens and forest birds.

Several investigations have supported the theory that wild birds play a small role in domestic bird disease (8, 22, 29, 30, 35). However, research into the details of disease transmission from free-roaming backyard chickens to wild birds is scant (14, 15, 24). The negative impact of backyard chickens on human health, especially as it relates to children, have been reported and are of concern, especially in developing nations (4, 16, 25, 26, 40).

Poultry production is a growing industry in Ecuador. Two new commercial and several small-scale poultry operations have been developed in the vicinity of the Maquipucuna Reserve. The poor husbandry practices often used in backyard flocks make these flocks a potential reservoir for diseases that can affect commercial poultry operations, especially diseases that have become rare in these operations(18, 43) . Investigations that effectively link human, animal, and ecosystem health are rare but should be supported. The objective of this study was to investigate the diseases of free-roaming chickens in northwestern Ecuador and is presented here as the preliminary phase of a larger effort in multidisciplinary conservation medicine.

MATERIALS AND METHODS

Owner surveys and animal examinations

Ninety-nine chickens (*Gallus domesticus)* and one turkey (*Meleagris gallopavo gallopavo*) that belonged to 10 private individuals were randomly captured for examination and blood collection during a 10-day period in December, 2003. Twenty-three animals belonged to an organic farm managed by the Maquipucuna Foundation and were kept in a fenced yard. The rest were free-roaming domestic birds living in the town of Santa Marianita, Ecuador, or in farms on the outskirts of the town. The town of Santa Marianita is located in the Pichincha province in the Northwestern portion of Ecuador, 4 km outside of the southwestern edge of the Maquipucuna Reserve and at 1300 m elevation. It is the last town where tourists can stop before entering the Maquipucuna Reserve and Ecolodge. The population of Santa Marianita is approximately 225 members, with most families living in a central area dedicated to residences and others living in surrounding farms. The landscape surrounding the village is composed of active agricultural land (pasture, sugar cane, and fruit trees) or low montane forest in varying stages of regeneration. All birds sampled were numbered sequentially from 1 to 100. Of the chickens sampled in this study, 45 came from dwellings in the center of village (approximately 20 dwellings in a semicircle surrounding a soccer field) (Figure 1). The remaining chickens sampled came from flocks in surrounding farms (Figure 2). All of the farms were within a 2-km radius of the village proper. The local chicken breed is a "creole" native red chicken, although 1 chicken examined was a silkie and 5 were Transylvanian naked necks.

All chickens were examined before samples were collected. A physical examination was performed to gauge body condition by palpating the pectoral musculature (scored from 1-5/5; 1=emaciated, 2=thin, 3=ideal, 4=overweight, 5=obese), subjectively assess the presence and degree of mite infestation (scored as: low = < 50 mites/one wing; moderate = 50-150 mites/wing; or severe = > 200 mites/wing), and to detect any notable abnormality. The same researcher scored all animals in this study to avoid sampling bias. Birds with a body condition of 3/5 or less or with severe mite infestation or other physical abnormalities were considered abnormal. The number of abnormal birds per flock was recorded. Ectoparasites were collected and immersed in 70 % ethanol for later identification based on morphologic characteristics by a parasitologist. Approximate age of each bird was estimated based on spur length and information provided by the owner according to the following scale: spur length < 1.3 cm (1/2 inch), juvenile bird; spur length 1.3-2.5 cm (1/2 to 7/8 inch), 1-year-old bird; and spur length ≥ 2.5 cm (7/8 inch), 2-year or older bird. Approximate age, breed, and sex were recorded for each bird.
All owners were interviewed during the sampling procedure by the primary author. Standardized survey questions were source of chickens; the total number of chickens; purpose for maintaining chickens; age at time of slaughter or sale; if chickens were penned at any time; approximate mortality rate; description of the clinical signs perceived as associated with disease; perceived needs for improving production, vaccination history, and nutrition; and whether any medication was administered to chickens. Although all chickens appeared to roam freely throughout the village and surrounding areas, each owner claimed they could recognize their own animals. **Sample collections**

Blood samples (3 ml) were collected from each of the 99 chickens and 1 turkey from either the right jugular vein or the superficial ulnar vein. One blood smear was made immediately, and the remaining blood was placed in a sterile clot tube. The blood smears were dried, stained with Wright's stain, and stored. The blood samples were maintained in a cooler with ice and centrifuged no more than 8 hours after collection. After centrifugation, approximately 1.5 ml of serum was transferred to cryovials and frozen at --18 C.. Blood products were prepared for importation following United States Department of Agriculture (USDA) guidelines for pathogen inactivation.

At least 5 fresh fecal samples were collected from each flock. Fifty fecal samples were examined for parasites by direct smear and fecal flotation on the day of collection, then discarded. Standard flotation techniques with sodium nitrate and Sheather's sugar solutions were followed.

Blood smears were examined for the presence or absence of hemoparasites by examining the entire blood smear at 100 X magnification to detect microfilaria then examining the monocellular layer at 400X. To determine disease seroprevalence, commercial enzyme-linked immunosorbent assays (ELISA) (Idexx Inc., West Brook, ME, USA) were performed for infectious bursal disease (IBD, gumboro disease), avian encephalomyelitis virus (AE, Picornaviridae), chicken anemia virus (CAV, Circoviridae), Newcastle disease virus (NDV, Paramyxoviridae), avian influenza (Orthomyxoviridae, type A), avian infectious bronchitis (IBV, coronavirus), *Mycoplasma gallisepticum*, and *M. synoviae* (University of Georgia Poultry Diagnostic and Research Center (PDRC), Athens, GA).

Sample-to-positive (S/P) ratios were calculated from absorbance values by the formula:

$$\text{S/P ratio} = \frac{\text{Sample mean - negative control mean}}{\text{Positive control mean - negative control mean}}$$

The S/P ratio is then converted to the antibody tier by a computer program developed by the manufacturer. Results were considered positive if the S/P ratio was > 0.2 for Newcastle disease virus, infectious bursal disease, infectious bronchitis virus, avian enchphalmyelitis virus or > 0.5 for *M. gallisepticum, M. synoviae,* and avian influenza. For chicken anemia virus, results were considered positive when the optical density was < 1.08. Capture ELISA to detect IgM and IgG against West Nile virus was performed at the University of Georgia Tifton Veterinary Diagnostic Laboratory.

Necropsy Examinations

Ten chickens that belonged to 3 different owners and that were not part of the previously sampled group were examined and humanely euthanatized. Necropsy was performed immediately after euthanasia. For each bird, a representative sample of organs (skin, muscle, nerve, trachea, thyroid, thymus, lung, air sac, heart, liver, spleen, kidney, adrenal, gonad and accessory structures, eye, brain, pancreas, esophagus, proventriculus, ventriculus, small and large intestine, cloaca, bone) were collected and preserved in 10 % buffered formalin for importation according to USDA Guidelines for pathogen inactivation. Parasites were collected into 70 % ethanol for transport and subsequent identification. Blood smears, parasites, and tissue and serum samples were imported into the United States under a zoosanitary certificate, issued by the Ministry of Agriculture

and Livestock of Ecuador (# 051643), and a USDA permit for importation of controlled materials, organisms, and vectors (# 46302 and # 27556).

RESULTS

Surveys

Private individuals maintained chickens primarily for eggs, personal use of the meat, breeding, and cockfighting. Mean flock size was 20 birds (range 1-75). None of the chickens examined in this study were cockfighting roosters; however, these roosters often foraged or were housed in contact with the free-roaming chickens. In general, owners reported that they bred their own chickens and kept most of the offspring; however, in some instances, owners mentioned that they traded or sold chickens with nearby villages. Chickens were slaughtered at 36 months or sold at 24 months. Fighting cocks were often traded or sold. None of the owners maintained formal records; however, all recognized which animals belonged to them. The area where their chickens foraged was reported to be between 200-300 m away from their respective homes. Owners captured and penned hens only when they were broody and thus incubating fertile eggs. Otherwise, except for fighting cocks, which were penned some of the time, chickens were not provided with permanent housing. Birds roosted in trees or on building structures at night. The chickens primarily foraged for food in active agricultural land and surrounding forest but were also supplemented daily with corn and, rarely, commercial poultry rations. Only fighting cocks were fed poultry rations regularly.

The disease syndromes that were perceived as the most important and that caused mortality in the flocks were: 1) “mal de pollo” (chicken plague), described as an acute disease during which most chickens in the area died suddenly or exhibited neurologic signs and which owners perceived as contagious and capable of spreading from flock to flock; 2) skin lesions, described as wart-like lesions on the head, face, and legs of the birds that were most prevalent during the dry season but caused little mortality; 3) “ronquera” (rales), a respiratory syndrome in which chickens exhibited mucopurulent discharge from the nares, excessive tearing, gasping, loud respiratory sounds, weight loss, and in some cases, death; and 4) a syndrome in which chickens became pale, lost weight, and died. Owners reported an overall annual mortality rate of between 35-50 %. A 50 % mortality rate was reported in chicks in the first 4 weeks of life, with diarrhea and respiratory signs cited as the top 2 reasons for death. Most owners (8/10) reported that they did not vaccinate at all. One owner reported vaccinating chickens when an outbreak of “mal de pollo” was occurring in the village; however, the specific vaccine used was not known. A second owner vaccinated annually with the same product. Although the particular product was not available for inspection at the time of this study, the local supplier was questioned and confirmed that Newcastle disease vaccine was sold to village owners. None of the owners treated their chickens for ecto- or endoparasites.

The percentage of abnormal physical findings in the chickens from each flock varied from 4 - 25 %. The owner with the largest flock (75 chickens) had the lowest number of abnormal animals. Abnormalities found were: moderate to heavy mite infestations (14%); thin body condition (10%); rhinitis (5%); poor feather condition (5%); evidence

of previous pox lesions on head, face, or legs (4%); increased respiratory effort (3%); and conjunctivitis (1%).

Serologic test results

In twenty-one (21 %) birds, antibody levels were above the seropositive threshold for avian influenza. Of these, 1 (S/P ratio 5.209) was likely caused by system error due to a dirty sampling cell (possibly from bacterial growth). In 6 samples, the S/P ratios were slightly above 0.500 (0.526-0.575). In 10 samples, the S/P ratios were higher (0.634-0.993); however, all of these results were lower than those in birds infected with avian influenza. In 4 samples, results were typical of birds infected with avian influenza (1.081-1.756). Therefore, 16 of the 21 samples had low S/P ratios and were considered negative.

Of the 100 birds tested for antibodies, 100 (100 %) were positive for infectious bursal disease, 85 (85 %) for infectious bronchitis virus, and 97 (97 %) demonstrated antibodies for Newcastle disease virus. Ninety-two (92 %) showed antibodies (titers >1500) for avian encephalomyelitis, with no difference in seroprevalence among flocks. In 90 (90 %) birds, antibodies were detected for chicken anemia virus. Seventy-three (73 %) had antibodies against *M. gallisepticum*, whereas 68 (68 %) had antibodies against *M. synoviae*. Antibodies against *M. gallisepticum* or *M. synoviae* were not present in one of the flocks tested. A histogram summarizing titer levels of each disease for all animals is presented in Figure 3.

Parasitology

No hemoparasites were found in the 100 blood smears examined. Parasites collected at necropsy were identified as cestodes (*Raillietina* species) and ascarids

(*Ascaridia* species). Two species of ectoparasites were collected and identified as *Dermanyssus gallinae* and *Ornithonyssus bursa*. On direct examination of fecal samples, 2 samples were positive for parasites: 1 contained strongyle-like eggs and the other contained coccidian-like cysts.

Necropsy and histopathologic results

Of the 10 birds submitted for necropsy, 9 were in fair to good body condition. Tapeworms were present in the upper and middle third of the small intestine in 8 birds. One bird exhibited hemorrhagic enteritis, and 1 bird had splenomegaly. In 2 birds, the liver had pale streaks throughout the parenchyma. One animal was extremely thin and had wart-like lesions on its head, severely thickened air sacs, a consolidated right lung, and a large number of tapeworms and ascarids.

All 10 chickens had mild, nonspecific infiltrates of few lymphocytes and plasma cells in various organs, such as liver, kidneys, heart, and lungs. These infiltrates were very minimal, similar to those seen in commercial poultry and not indicative of any particular disease. Specific lesions observed in all chickens were marked infiltrates of lymphocytes and plasma cells in the mucosa of the small and large intestine, sometimes accompanied by loss of crypts in both small and large intestine and mild atrophy of villi in the small intestine. Germinal centers were also occasionally seen in small and large intestine. Numerous sarcomastigophoran protozoa were present in the large intestinal crypts in 7 birds. These protozoa appeared amoeboid, were 10 - 25 microns in diameter, and were confined to the crypt lumen. Morphologically, protozoa were most consistent with an amoeboid, *Entamoeba*-like organism.

Other parasites were found in several birds. Histologic evidence of nematode infection was found in the proventriculus, ventriculus, small intestine, or cecum in 4 chickens. Inflammation in the wall of the ventriculus in 1 bird was consistent with previous parasite migration. Cestodes were also confirmed histologically in the intestinal lumen of 2 chickens. One chicken had numerous sarcocysts in its skeletal muscle, 1 had respiratory mites identified histologically, and 1 had evidence of pulmonary nflammation with mite debris in its lungs.

One chicken had an enlarged spleen due to the presence of numerous adenoviral inclusions in the nuclei of splenic macrophages. These inclusions were large, lightly basophilic, and compressed the chromatin to the periphery of the nucleus. These inclusions were consistent with infection with a group II avian adenovirus, such as the viruses that cause hemorrhagic enteritis in turkeys, marble spleen disease of pheasants, and adenovirus-associated splenomegaly in chickens. Although these viral infections may cause serious disease, particularly in young birds, this chicken appeared relatively healthy and was reproductively active. Histopathologic examination of the wart-like skin lesions revealed proliferative epithelial cells exhibiting ballooning degeneration with eosinophilic intracytoplasmic inclusion bodies, confirming a diagnosis of avian pox. Another chicken had severe, chronic bacterial pneumonia due to a small bacterial rod, most consistent with an enteric organism such as *Escherichia coli* or possibly *Pasteurella multocida*. Histologic evidence of a preexisting lower respiratory tract disease was not present in this chicken; therefore, the infection was likely primary, and not secondary, to another infection such as Newcastle disease or mycoplasmosis. Other than the 2 isolated findings of adenoviral splenitis and bacterial pneumonia and evidence of parasitism,

avian pox, and chronic enterocolitis, no other significant disease was evident histologically in this group of 10 chickens.

DISCUSSION

In this study, backyard poultry in Ecuador showed evidence of exposure to important poultry pathogens. These disease pathogens, both singly and in combination, are likely responsible for the high mortality of young birds and potentially for decreased reproductive success of adults. Possibly, chickens from other towns that are sporadically purchased or introduced into backyard flocks act as disease reservoirs, and this practice may relate to the epizootics reported by owners. In general, production in these free-roaming flocks is poor. The overall high mortality rate reported by owners indicates that the chickens would benefit from a detailed preventative medicine protocol. Peer-reviewed sources that outline protocols for backyard flocks, especially those that consider cost:benefit ratios for the owners, are rare (18). Given the potential hazard to human and wildlife health, as well as threats to commercial operations, a thorough review of backyard flock preventative medicine is long overdue.

Preventative medicine in chickens has been proved to decrease production loss and mortality (34). However, lack of education and cost of preventative measures preclude the owners in this village in Ecuador from applying standard preventative medicine protocols, increasing the susceptibility of the chickens in their flocks to a variety of bacterial, viral, and parasitic diseases. The high mortality rates reported are directly linked to the lack of preventative medicine and shelter, the practice of keeping chickens

of different ages in the same group, and allowing new chickens to be introduced into existing flocks in the village.

The liquid vaccine used by 2 owners was likely a vaccine prepared for Newcastle disease, as this is the most commonly administered vaccine for poultry available in Ecuador. However, it was inappropriately stored and administered and thus unlikely to have been effective. Five months after this project was completed, another outbreak caused sudden deaths in more than 30 chickens after chickens from Quito were introduced. The owners described it as “mal de pollo”. Because our survey did not take place during an outbreak, we did not perform diagnostic tests to investigate the cause. However, considering the history and clinical signs, the high prevalence of Newcastle antibodies among this population of chickens, the high antibody titers against Newcastle disease virus, and results of consultation with government veterinarians and poultry veterinarians with experience in Ecuador, we concluded that this ‘plague’ was most consistent with an outbreak of Newcastle disease. Indeed, in the older literature, Newcastle disease is referred to as “chicken plague” and, in some Spanish-speaking countries, Newcastle disease is often termed as “plague”.

Although avian pox was confirmed only in 1 chicken, our survey was conducted during the rainy season, a time when owners reported the lowest prevalence of wart-like lesions. We are confident that avian pox is a likely diagnosis for the aforementioned skin lesions. Given the results of serologic tests and questionnaires, the respiratory disease described by owners likely was caused by either Newcastle disease virus or infectious bronchitis virus. It is more difficult to speculate on the last of the 4 major diseases causing significant mortalities in these flocks, described as causing anemia, weight loss

and death. Tumors caused by Marek's disease or leucosis might explain these clinical signs.

Most chickens would be expected to have been exposed to avian encephalomyelitis, infectious bursal disease, infectious bronchitis virus, chicken anemia virus, Newcastle disease, and *M. gallisepticum*/*M. synoviae* . The USDA lists Ecuador as a country with endemic exotic Newcastle disease as well as the other typical poultry diseases (1). The positive titers on ELISA tests indicate that these chickens were exposed to these diseases at some point and survived the infection. None of the birds examined exhibited clinical signs consistent with these diseases; therefore, no definitive comment can be made about whether the chickens expressed clinical disease after exposure. However, usually an antibody titer can only result from exposure to those particular pathogens and not from maternal antibodies or previous vaccination, particularly for Newcastle disease, infectious bursal disease, infectious bronchitis, and *Mycoplasma* species.

Given the significance of avian influenza, the results obtained in this study were carefully examined. The threshold for a seropositive result for the ELISA as determined by the manufacturer is 0.500 S/P. Of the 21 positive samples, 1 was likely laboratory error. In 6 samples, S/P ratios were slightly above the threshold value and unlikely to be true positives. In 10 additional samples, S/P ratios were not as high as those usually seen in birds infected with avian influenza. Therefore, only 4 samples had results typical of birds infected with this disease. However, given the highly transmissible nature of avian influenza, it is unlikely that 4 birds would be infected without the rest of the flock also exhibiting seroprevalence. Current reports of avian influenza outbreaks demonstrate seropositive rates greater than 90% in infected flocks. To further confirm the presence of

antibodies for avian influenza in the seropositive birds, agar gel immunodiffusion (AGID) testing could be done; however, this was not done in our study because of insufficient amount of serum.

There was only one significant difference in disease seroprevalence among flocks. One flock, located 2 km from the village, showed no seroprevalence to *M. gallisepticum* or *M. synoviae*. The chickens in this flock were not in contact with chickens from other farms or village. Indeed, even in commercial operations, the seroprevalence of *Mycoplasma* can vary, with some flocks having 0% seroprevalence (1). Seroprevalence rates for the remaining diseases did not differ among flocks, indicating that those birds kept in more isolated farms were exposed to the same diseases as birds kept within the village proper.

Free-roaming chickens are commonly exposed to a variety of parasitic diseases. In young birds, heavy infections of cestodes can result in reduced efficiency and slower growth (1). Poultry become infected by ingesting the intermediate hosts of cestodes such as snails, slugs, beetles, ants, grasshoppers, earthworms, houseflies and other arthropods. The intermediate host becomes infected by eating the eggs of tapeworms that are passed in the bird feces. The host of *Raillietina* species is a beetle, and chickens foraging have ample opportunity to ingest this and other arthropods. *Ascaridia* species, the largest internal nematodes that infest the small intestine, can cause poor body condition and intestinal impaction. Heavy infestations can cause death. Chickens 3 to 4 months of age show resistance to infection (1, 34). The worm is transmitted vertically from hen to offspring; therefore, infected adults can be source for young (34). Additionally, embryonated ascarid eggs are very hardy and under laboratory conditions may survive for

2 years. However, in field conditions, few probably survive more than 1 year (34). Chickens become infected by ingesting eggs that have reached the infective stage. At necropsy, most chickens that were examined had either no or low numbers of ascarids in the intestines. One chicken that had a heavy load of ascarids also had severe pneumonia. Fecal flotation examination yielded a very small number of parasites per sample and in total.

In general, intestinal parasitism was not a major concern in this group of chickens. This was expected because, as opposed to commercial poultry operations, chickens in free-roaming conditions are not concentrated in small areas, do not frequently come in contact with the fecal material of other chickens, and are not confined to contaminated bedding. Parasites that require an intermediate vector were observed in these birds but was not of major concern. It is difficult to ascertain how the ectoparasite infestations observed in these birds affected their health. In commercial operations, ectoparasites can affect production, but because production is not monitored in these flocks, it could not be correlated with levels of ectoparasites.

On histopathologic examination, the marked infiltrates of lymphocytes and plasma cells identified in the mucosa of the small and large intestine indicate that these chickens probably survived an episode of enterocolitis, resulting in the residual inflammation. A specific cause was not observed in any chicken. The amoeboid protozoa found in the intestine, morphologically consistent with Entamoebae, have been described previously parasitizing the intestinal tract of chickens, but their significance is uncertain (34). Because amoeboid protozoa were present in only 7 birds, they are not likely to be the only cause for the prominent intestinal inflammation in all birds. The chronic

enterocolitis could indicate previous exposure to other intestinal pathogens, such as *Salmonellae*; however, this was not confirmed. Further investigation into the bacterial pathogens, including microbiologic culture of fecal samples of these chickens, is warranted.

Compared with commercial poultry, free-roaming chickens are both at an advantage and disadvantage for maintaining health. Free-roaming chickens do not receive vaccinations typically given to commercial poultry, including vaccinating hens to increase maternal antibody transferred to chicks. This renders free-roaming chickens inherently more susceptible to many infectious diseases. Additionally, free-roaming chickens are not given treatments commonly used in commercial poultry, such as coccidiostats. Free-roaming chickens are unlikely to be on the same nutritional plane as commercial birds, which are provided a complete, balanced, pelleted diet. Commercial birds are maintained in single age groups in an "all in, all out" manner, whereas free-roaming chickens are in flocks of mixed ages, with susceptible young chicks in contact with adults that are potential reservoirs of disease. Therefore, an infectious disease could easily be maintained in a free-ranging flock by a continuous supply of new susceptible hosts coming into contact with reservoir animals. Lastly, most commercial poultry breeder flocks are maintained free of certain infectious diseases that can be transmitted from the hen to her progeny, including *Salmonella pullorum, S. gallinarum, M. gallisepticum*, and *M. synoviae*. Because free-roaming chicken flocks are not monitored for these diseases, diseases could remain endemic in the population through continued egg transmission.

Free-roaming chickens have the advantage of not being reared or maintained in confinement at the intense stocking densities that are typical of commercial poultry. In some commercial poultry operations, birds are commonly reared and maintained on used litter that potentially harbors pathogens. Certain diseases in commercial birds, such as coccidiosis and Marek's disease, are perpetuated by these management practices (34). These diseases would be expected to have little impact on free-ranging chickens because they are not exposed to contaminated litter and dander in a confinement situation.

The diseases for which these birds were tested have significant economic importance in the poultry industry (34). Commercial poultry is a rising industry in Ecuador. Two commercial poultry operations are located near the town of Santa Marianita, and although biosecurity is stringent in these operations, it is important for the Ecuadorian authorities to be aware of the diseases harbored by free-roaming chickens in case of a biosecurity breach that might lead to an epizootic.

In this study, the only zoonotic disease agent detected was Newcastle disease virus, which can cause photophobia and transient conjunctivitis in people (34). However, the bacterial diseases of this group and similar flocks of chickens should be investigated because bacterial agents such as *Salmonella* species and *Campylobacter* species are more important zoonotic diseases. Additionally, salmonellosis has been implicated as an emergent disease of wild birds (37).

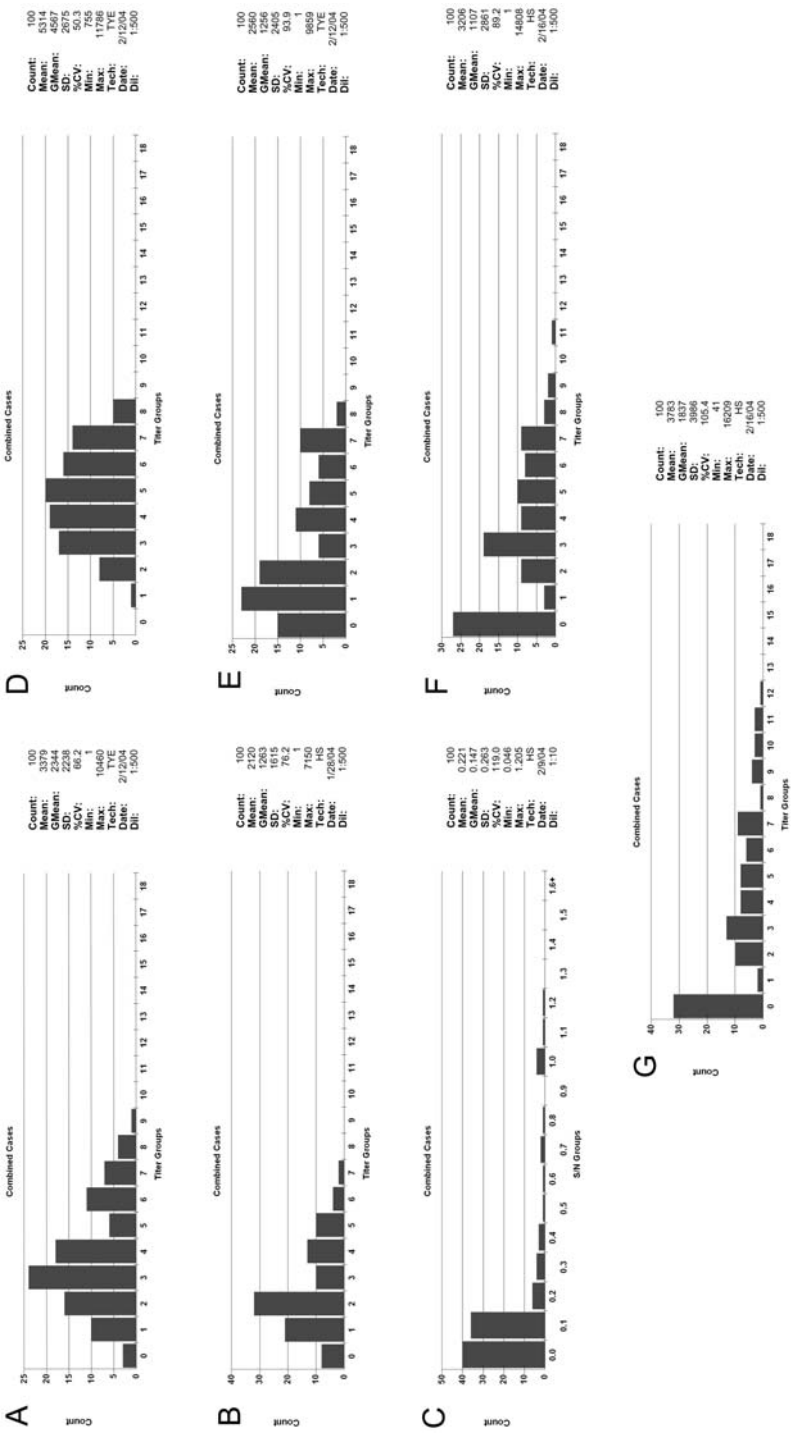
The susceptibility of wild birds to poultry diseases is unknown. There are several reports of Newcastle disease and highly pathogenic avian influenza infecting wild birds(15, 21, 38, 39). “Pathogen pollution” is a term applied to the anthropogenic introduction of pathogens into new areas (9). It is impossible to know if these avian

viruses and parasites were present in the area before the town was settled and domestic birds were introduced or if they present a significant threat to the native avian populations. At this time, no deaths have been observed in the wild bird populations of the area; however, it is often very difficult to discover dead wild birds in a remote tropical forest unless hundreds or even thousands have died. Therefore, investigating the risks that free-roaming birds and their endemic pathogens pose to wild birds would be prudent.

Figure 1. A survey of selected pathogens from backyard chickens was performed in the town of Santa Marianitas, located in the Northwest of Ecuador. The village proper, from which 45 chickens were selected for the survey, is depicted here.

Figure 2. Farms outside of the village proper, but still part of the town of Santa Marianitas, were also chosen for a survey of selected pathogens affecting their backyard chickens. This farm is located approximately 2 km from the village proper and is surrounded by agricultural and forested landscape.

Figure 3a-g. Distribution of antibody titers to infectious diseases determined by ELISA on serum samples from 100 chickens in Ecuador. (A, Newcastle disease virus; B, avian encephalomyelitis; C, chicken anemia virus; D, infectious bursal disease; E, infectious bronchitis virus; F, *Mycoplasma gallisepticum*; G, *Mycoplasma synoviae*).



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CHAPTER 4

BACKYARD CHCKEN FLOCKS POSE A DISEASE RISK FOR NEOTROPICAL BIRDS IN COSTA RICA

INTRODUCTION

In 2001, Friend detailed examples where recent disease emergence has had significant effects on wild bird populations, and made a plea to avian and conservation communities to support more proactive, comprehensive examinations of risk factors that could affect further emergence of diseases (23). In response to his recommendations, we aim to understand how specific human-altered systems, and introduced avifauna, can affect disease dynamics for wild birds. Costa Rica is one of the hotspots of biodiversity of the world (54). The highlands of the Republic of Costa Rica harbor the greatest avian species richness in Central American montane forests and one of the highest levels of avian endemism in the world (35). The Monteverde region, in the Northwestern part of the country, is the second top eco-tourist destination, attracting visitors specifically seeking its natural beauty and rich avian biodiversity (6). However, outside of protected reserves, the landscape continues to be deforested for agricultural use, an activity that threatens the status of neotropical migrants and resident avifauna in a variety of ways. Preservation of avian biodiversity in this region should therefore be a priority, both from an economic and conservation stance.

One conservation incentive heavily promoted is the creation of shade-coffee parcels, considered forest surrogate habitat, as they provide floristically and structurally diverse habitat, positively affecting avian biodiversity. Nevertheless, they might also pose disease risks by artificially concentrating and aggregating birds to areas rich in food resources and exposing them to invasive species—such as the domestic chicken. We hypothesized that free ranging backyard chickens could serve as disease reservoirs for susceptible wild bird populations.

Backyard chicken flocks have began to receive attention due to their role in the epidemiology of avian influenza in Asian countries and are currently being closely scrutinized in many countries (8, 10, 69, 70, 73). Additionally, game chicken flocks have been involved in outbreaks of economically significant diseases, such as Newcastle Disease (NDV), leading to the slaughter of many birds and expensive biosecurity efforts (12, 27). Although the exports of poultry products by Central American countries are small, and mostly confined to trade within the region, Costa Rica is Central America's principal poultry exporter, and currently marketing to the US, explaining Costa Rica's efforts to be declared Newcastle disease-free (1). Backyard flocks can act as potential reservoirs for diseases that can affect commercial poultry operations, especially of diseases that have become rare in these operations (40). A recent review by the USDA has recommended the need to examine backyard chicken flocks near commercial operations, more closely (52). Despite disease concerns, and that backyard chicken flocks in this study are typical of the way chickens are maintained across the developing world, there is a paucity of published information regarding the pathogen prevalence and diversity of backyard chickens, particularly in Latin America (31, 33, 36, 42, 59, 61).

According to the Costa Rican Poultry Association (A.E. Musmanni, pers. comm., 2007), large commercial poultry operations are well established in Costa Rica, but backyard flocks are still very common, with a majority of families in rural areas primarily dependent on poultry for their sustenance. Poor preventative medicine negatively affects production, and information on the health and disease status of backyard chicken flocks is needed to generate recommendations that benefit rural communities.

Here we present the results of a disease survey conducted with the objective to determine if free ranging backyard chickens inhabiting shade-grown coffee parcels pose a source of pathogens for wild birds that share these habitats. In addition, we aimed to determine the baseline antimicrobial resistance pattern of fecal bacterial isolates as a model to study microbial distribution patterns and transmission in this habitat. During the course of the study, it became obvious that the health and disease prevalence of backyard chickens is also important to people participating in sustainable agroforestry incentives, and nearby commercial poultry operations.

MATERIALS AND METHODS

Study area

The study took place in the town of San Luis (10 16'57,117" N 84 47'53,747" W), 7 km South West of the well-known Monteverde region in Northwestern Costa Rica and housing approximately 60 rural families. The landscape of this region is comprised of a small residential area, a cooperative farm that contains shade-grown coffee plantations, pasture, and a mixture of primary and secondary tropical, premontane forest fragments. The flocks selected for this study were either located within

shade-grown coffee plantations, or were immediately adjacent to such plantations (Figure 1).

Study subjects, Interviews and Examinations

One hundred and fifty-one chickens (*Gallus domesticus)* from 13 flocks were randomly captured for physical examination and biological sample collection during three separate time periods July 2005, November 2005, and February 2007. All chicken owners were interviewed in Spanish during the sampling procedure by the primary author.

A physical exam was performed to gauge body condition by palpation of the pectoral musculature (scored from 1-5/5; 1=emaciated, 2=thin, 3=ideal, 4=overweight, 5=obese), subjectively score presence and degree of mite infestation (scored as low=less than 50 mites per one wing, moderate=50-150 mites per wing, or severe=greater than 200 mites per wing), or any notable abnormality. If animals had a body condition of 2/5 or less, showed severe levels of mites, or any other physical abnormality or clinical signs they were considered abnormal.

Biological Sample collection and Disease Surveillance

Blood was collected from 151 chickens. A thin blood smear was made immediately, dried, stained with Wright's stain, and examined for the presence or absence of hemoparasites (68). At least five fresh fecal samples were collected from each flock, preserved in a 2.5% potassium dichromate solution and examined microscopically, first directly and subsequently by standard flotation technique with Sheather's sugar

solution (68). Ectoparasites were collected and stored in 70% ethanol for later identification using morphologic characteristics. Serum was collected and maintained at -80° C until processing. Serum samples were tested using a commercial enzyme-linked immunosorbent assays (ELISA; Idexx Inc., West Brook, ME, USA) for Avian Pneumovirus (APV), Infectious Laryngotracheitis (ILT), Infectious Bursal Disease (IBD), Avian Encephalomyelitis virus (AE), Chicken Anemia virus (CAV), Newcastle Disease (NDV), Avian Influenza (AI), Avian Infectious Bronchitis (IBV), *Mycoplasma gallisepticum*, (MG) and *M. synoviae* (MS) at the School of Veterinary Medicine, Universidad Nacional de Costa Rica or at the University of Georgia Poultry Diagnostic and Research Center (PDRC), Athens.

Choanal swabs were collected from 21 birds for *Mycoplasma gallisepticum* and/or *M. synoviae* nucleic acid detection. DNA was extracted utilizing a commercial available Qiamp Mini kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. The DNA was frozen at -70° C until processing. *Mycoplasma gallisepticum* DNA was detected with a Real-time Taqman® polymerase chain reaction (R-PCR) and *Mycoplasma synoviae* DNA was detected with a PCR assay described by Lauerman *et. al* (9, 46).

Cloacal swabs from nine birds from three flocks were collected and prepared for virus isolation by inoculating chickens embryos. The allantoic fluid was tested for hemmaglutinating activity and the samples were placed on FTA cards (FTA© classic cards, Whatman International Ltd, Springfield Mill, James Whatman Way, Maidstone, Kent, UK) for the molecular detection of Newcastle disease virus, as previously described (57).

Fresh fecal samples were collected and fecal bacteria propagated on MacConkey media plates, incubated at 37° C for 12-18 hrs. Individual colonies were introduced into stab storage media (tryptone 0.2 %, yeast extract 0.02 %, and agar 0.5 % in distilled water) and maintained at 4° C until export. Once in the USA, bacteria was streaked for re-isolation on to both blood and MacConkey agar plates and incubated at 35±2° C for 12-18 hrs to confirm purity. Bacterial identification was done through standard biochemical reactions (triple sugar iron, motility, indole, ornithine, oxidase, and citrate) or with commercially available *Enterobacteriacae* identification strips (API 20E; bioMerieux USA, Durham, NC). Minimum inhibitory concentrations (MIC) were determined using TREK, diagnostic system plates following the manufacturer's instructions (11). MIC plates contained a series of titrations of 13 different antibiotics (Trek Diagnostics, http://www.trekds.com/products/sensititre/vet_ssmic.asp, Cleveland, OH). Resistance breakpoints were determined based on previously published data (11). Whole cell templates were made of pure culture stock of lactose fermenters (63). Polymerase chain reaction was used to identify the samples that contained drug resistance genes such as Class I Integron (*int1*), and t*et*(A) and *tet*(B) as previously described (4, 29, 55). A list of primers utilized is summarized on Table 1. Fourteen chickens, which belonged to 12 owners, were examined and humanely euthanized. A gross necropsy was performed immediately after euthanasia. For each animal, a representative sample of all organs were collected and preserved in 10 % buffered formalin until examination.

All biological samples were prepared for importation following USDA guidelines for pathogen inactivation, and were imported into the USA under a United States Department of Agriculture (USDA) import permit and a Ministry of Agriculture of Costa

Rica export permit. All of the work was approved by the University of Georgia's Institutional Animal Care and Use Committee.

RESULTS

Interviews and Examinations

Chickens were the responsibility of the women in the household and they were maintained primarily for personal use of the meat and eggs, and breeding. Five flock owners indicated they "sometimes" obtain some chickens from the Ministry of Agriculture (MAG). Chickens distributed by MAG are vaccinated against Newcastle disease vaccine, IBD, IBV, and pox virus. Chickens foraged within the boundaries of the owner's properties but frequently entered nearby farms or forest fragments throughout the day and were penned in rustic coops at night. The chickens primarily foraged for food, but were also provided with kitchen scraps, crops (i.e.: bananas) cracked corn and commercial poultry rations which did not contain antimicrobials or coccidiostats. None of the owners routinely vaccinate their chickens.

Owners reported an average 5-20 % mortality rate of chicks in the first 4 weeks, primarily from diarrhea and respiratory signs and an annual mortality rate of 0-17% in their adult birds. The disease syndromes owners perceived as the most important and that caused mortality in the flocks were a Newcastle disease-like syndrome, fowl pox, respiratory disease and an undetermined acute, anemia syndrome. Upon examination, the percentage of abnormal physical findings in the chickens from each flock varied from 43-99 %. Abnormalities found were: severe mite infestations (42%; range 28-100%); thin body condition (67%; range 14-100%); respiratory signs (13%; range 0-25%); evidence

of current or previous pox lesions on head, face, or legs (1%); loss of normal leg scales (100% in animals > 2 yrs of age).

Disease Surveillance

The results for NDV, ILT, IBV and APV, MG and MS are represented on Tables 2, and 3 respectively. Of the 128 chickens tested, 72 had an S/N ratio of 0.029-0.700 and were considered positive for CAV, while 56 had an S/N ratio between 0.700-0.921 and were considered negative. Of the 151 ELISA extended range titers acquired for IBD, 33 were <1,000; 17 were between 1,000 and 1,999; 41 fell between 2,000 and 3,999; and 60 were > 4,000. Interpretation of antibody titers depends on many factors such as type of vaccine (e.g. live vs. inactivated), level of challenge in the field, and host immune status. Based on previous experience with the clinical significance of antibody titers in poultry, and the test manufacturer's recommendations, we grouped the titer results into categories such that <1,000 were considered negative or very low; 1,000-2,000 was low to moderate; 2,000-4,000 was considered high, and >4,000 was very high. Additionally, 38 birds were tested against AE, of which 13 showed antibodies above the manufacturer's threshold against AE (34%); however, only two individuals (5%) from two separate flocks had titers > 1,500. One hundred and eighteen birds were tested for the presence of antibodies against AI, of which 12 (10%) showed the presence of antibody ranges above the manufacturer-recommended threshold for seropositivity (range: 26 to1495). None of these were confirmed positive by agar gel immunodiffusion (AGID). The hemagglutination test performed with the allantoic fluid obtained from embryos after three passages was negative for NDV. Additionally, paramyxovirus nucleic acid was not

detected via PCR in any of the samples tested. Twenty-one birds from six flocks were tested via Real-time PCR for *M. gallisepticum,* detected in six samples (29%), and regular PCR for *M. synoviae*, detected in 14 (67%) samples.

No hemoparasites were found in the 96 blood smears examined. Direct and flotation techniques of 65 fecal samples from 13 flocks yielded: 1) *Eimeria* oocysts (*E. tenella*, *E. acervulina*, *E. brunette*, and *E. necatrix*) in 46% of samples, 2) nematode ova identified as capillarids (54%), *Dyspharynx* sp. (8%), and *Ascaridia galli* (20%), and 3) cestode eggs consistent with *Raillientina* sp. (42%). More than one species of parasite was present in 86% of the samples examined. Two species of mites collected were identified as a wing mite, *Pterolichus obtusus*, (Robin; Family Pterolichidae) and a body mite, *Megninia cubitalis* (Megnin; Family Analgidae). The lice collected were the shaft louse, *Menopon gallinae* (Linnaeus; Family Menoponidae), a wing louse, *Lipeurus caponis* (Linnaeus; Family Philopteridae) and *Oxylipeurus dentatus* (Sugimoto; Family Philopteridae).

Of the fecal samples collected, only those that yielded bacterial isolates that were lactose fermenters were analyzed; therefore, the results discussed are from 48 isolates. The majority of lactose fermenter isolates were identified as *E. coli* (91.6%), and the remaining isolates were other genera in the group *Enterobacteriacae* (8.4%). The antibiotic resistance profile and the prevalence of gene presence are represented on Tables 4 and 5. Specifically, the presence of resistance-conferring genes in the 18 samples that displayed phenotypic resistance against tetracycline, are represented on Table 5. Also of significance, 14/44 (32%) *E. coli* isolates displayed intermediate resistance to cephalothin (16 μg/ml); 27/44 (61%) to florfenicol (4 μg/ml); 4/44 (9%) to

difloxicin; 2/44 (5%) to orbifloxicin (2-4 μg/ml); 41/44 (93%) displayed the lowest susceptibility for ceftiofur (≤ 2 μg/ml); 26/44 (59%) to amoxicillin with clavulinic acid (≤ 4/2 μg/ml), and 15/44 (34%) to cephalothin (≤ 8 μg/ml).

Fourteen chickens from 12 owners were submitted to complete pathology examinations. In general, all animals were found to have moderate-severe ectoparasite infestations (mites, lice), evidence of mild to moderate endoparasitism, poor body condition, and birds older than 1 yr of age were found to have moderate-severe dermatitis of the lower legs due to *Knemidocoptes sp.* infestations. Parasites found during gross necropsy and microscopic examinations were consistent with *Capillaria* spp, *Ascaridia* spp, *Heterakis* spp, protozoal organisms consistent with *Eimeria necatrix*, and flagellates consistent with *Tetratrichomonas gallinarum*. Significant gross and microscopic findings included: 1) subcutaneous heterophilic granulomas containing branching hyphae, of which pure, heavy growth of *Aspergillus clavatus*, *A. flavus* and *A. fumigatus* was cultured (n=2), 2) hyperplastic epithelium with ballooning degeneration and large eosinophilic intracytoplasmic inclusions consistent with poxvirus (n=2), 3) lymphocytic sinusitis highly suggestive of *Mycoplasma gallisepticum* (n=1), 4) splenic lymphoid atrophy, suggestive of immunosuppressive agents (n=2), 5) ovarian adenocarcinoma (n=1), 6) anthracosis (n=10), 7) lymphocytic myocarditis and epicarditis suggestive of reovirus (n=2). Minor findings included: 1) protozoal typhlitis (n=4), and 2) mild-moderate parasitic enteritis (n=8).

DISCUSSION

Free-roaming chickens are at a disadvantage compared to commercial poultry for maintaining their health, as they do not receive vaccinations or are afforded treatments typically applied to commercial poultry. These chickens are on a poor plane of nutrition and run in flocks of mixed ages, placing susceptible younger chicks in contact with adults that are potentially reservoirs of disease. Additionally, most commercial poultry breeder flocks are maintained free of certain infectious diseases that can be transmitted from the hen to her progeny. It is likely that the diseases, to which these chickens are exposed, both singly, or in combination, are responsible for high mortality of young, and potentially decreased reproductive success. On the other hand, their relative low densities, hybrid vigor and capability to free-roam away from excrement may prevent them from suffering from more substantial clinical disease. Lack of education, geographic isolation, and cost of veterinary services precludes the owners in this community from applying standard preventative medicine protocols. Morbidity and mortalities reported are directly linked to the lack of preventative medicine, the lack of shelter, keeping chickens of different ages in the same group and allowing the introduction of new individuals into the flocks (5). Owners reported sporadic deaths due to a “chicken plague”, which, based on the history and clinical signs, high prevalence of NDV antibodies among the population, and elevated antibody titers against NDV, led us to the conclusion that this is most consistent with an outbreak of Newcastle Disease.

At least one report indicated that poxvirus is not uncommon in backyard chicken flocks in the USA (32). Given the lack of vaccination, the presence of insect vectors,

and the clinical and microscopic examination findings in this study, we suspect that poxvirus will continue to be an important cause of decreased production of these flocks. Although pox viruses are typically host-specific, there is a possibility of recombination of chicken and passerine poxvirus strains, which could affect virulence.

Given our serology results and interviews, it is likely that the respiratory syndrome described by owners is caused by NDV, IBV, Mycoplasmosis or ILT. Tumors caused by Marek's Disease or Avian Leukosis or other conditions causing severe anemia might explain the other syndrome owners reported as anemia, weight loss and death, as at least one of the birds necropsied exhibited splenic atrophy, often associated with immunosuppressive agents like Marek's, CIA or IBD.

Given the significant number of birds with titers >4,000, we assume that backyard chickens in this region have been in contact with a pathogenic strain of NDV, even when taking into consideration that some birds might have received live vaccines. The USDA and the OIE considers Costa Rica a country free of exotic Newcastle Disease, but the status of backyard chickens is unknown (1). Worldwide, NDV has the potential to cause morbidy and mortality, and has been detected in a variety of wild birds (27, 30, 41, 43, 48). Of those relevant to this region, high level of susceptibility to NDV is reported in Galliformes, Psittaciformes and Columbiformes. Lesser susceptibility occurs in Falconiformes, Accipitiformes and Passeriformes. Gilchrist provides a recent comprehensive review of wild bird susceptibility to NDV (26). Of particular concern would be other members of the Galliformes, of which, certain species are frequently observed in shade-grown coffee plantations or immediately adjacent forest fragments, such as three members in the Family Cracidae, and one in the Family Odontophoridae. In

this region there are six Columbiformes inhabiting shade-grown coffee parcels, one of which is considered rare. Five species of psittacines, highly coveted by ecotourists, make use of coffee parcels. Due to the feeding ecology of the members of the Galliformes and Columbiformes, contact with chickens or their excrement is considered most likely. For example, the author has observed Inca doves (*Columbina inca*) and White-tipped doves (*Leptotila verreauxi*) feeding with chickens (67). Pigeon paramyxovirus (PPMV-1) antibodies were detected from a variety of wild birds with highest frequency during a regional outbreak of PPMV-1 in white collared doves (*Streptopelia decaocto)* in Florida, suggesting that this virus had spread to other wild birds. Both that study and Gohm's serosurvey of wild birds during an NDV epizootic illustrate the subtlety with which this virus circulates in natural populations (27, 71).

Close inspection of the birds with titers >4,000 for IBV indicated that they did not originate from flocks where owners reported acquiring birds from the MAG and we would consider those titers significant. Seropositivity against IBV has been reported in wild birds such as pigeons (3). Jimenez *et. al.* reported on the widespread distribution of IBV in Costa Rica, a similar prevalence (42%) in the backyard poultry they examined, and the prevalence of IBV antibodies in free-ranging Columbiformes, including species that routinely inhabit shade-grown coffee plantations (38). Again, aforementioned wild birds in the orders Galliforme and Columbiforme would be considered at highest risk.

Since these backyard chickens were not vaccinated against ILT, the 65 birds with titers >1,000 were likely previously infected with virus. Currently, Costa Rica is experiencing sporadic outbreaks of ILT in their commercial operations, in which more than 50% of the animals are seropositive (C. Jimenez, pers comm., 2007). Therefore, it

appears that ILT could also be a significant disease for this population of backyard chickens. ILT has been reported in members of Phasanidae and Numididae, but the susceptibility of their New World counterparts in the Cracidae and Odontophoridae families is unknown (39, 72).

Based on serology and PCR, these chickens were also infected with *Mycoplasma gallisepticum* and *M. synoviae*. It appears that *Mycoplasma* sp. diseases are very common in backyard chicken flocks (36). The DNA presence of Mycoplasma mimicked the antibody seroprevalence. All of the birds in which *M. gallisepticum* nucleic acid was detected were less than one year of age and although the sample size in this study would preclude us from definitively investigating the relationship between age and antigen detection, we speculated that juveniles harbored more *M. gallisepticum* organisms. At least one study states that lower quantities of *M. gallisepticum* DNA are found in older birds (24). *M. gallisepticum* causes respiratory disease in a variety of wild birds (22, 50, 51), and a variant associated with poultry and turkeys has caused population declines due to conjunctivitis and mortality of house finches and other members of Fringillidae (13, 18). There are 134 species of Passeriformes that inhabit this region of which at least 74 regularly inhabit shade coffee plantations and of those, 18 species forage primarily on the ground and could be considered at risk (Hernandez-Divers unpub. data). Twenty-five of the common Passeriformes in shade-grown coffee parcels are North American migrants that rely heavily upon surrogate habitat.

Since 56% and 40% of chickens had titers consistent with infection with CAV and IBD, respectively, it appears these diseases are also common in this population of chickens. Antibodies against IBD have been reported in a variety of wild birds (37, 56).

Of relevance to our study are birds in the genera *Corvus* and *Columba,* although antibodies have been found in other members of Passeriformes (26). As this virus causes immunosuppresion, clinical disease might be expressed in terms of secondary infections, leading to indirect causes of mortality (e.g. predation).

Multiple infections with a variety of parasites are common in free range chickens ((17, 33, 36, 40, 58, 61, 64, 65). Losses in weight, egg production and longevity of free roaming chickens due to parasitic disease might not be as apparent, when compared to viral or bacterial disease, but can be far more significant (34, 58, 65). Co-infections with three species of *Eimeria* were noted in high numbers on fecal exams and were associated with clinical disease in pathologic examinations. Coccidiosis can be a major cause of mortality among chicks and a cause of morbidity and loss of condition among adult chickens (53). Except for *Knemidocoptes sp.,* which caused visible irritation and dermatitis, the other mites and lice recovered are host-specific and not considered particularly pathogenic. A subtle, yet important factor affecting production is the interrelationship of parasitic infections and other diseases, and currently there is interest in understanding this relationship, (12, 16). Although parasitic infections are generally host-specific, some important exceptions exist. For example, in addition to members of the Galliformes, both *Dyspharynx* sp., *Capillaria* sp., are nematodes that have the capability of infecting a variety of Passerine hosts (20, 60). Although nematodes found in chickens have always been considered host-specific, for example, *Syngamus trachea* has been reported in a variety of wild birds (44). In fact, preliminary data has demonstrated *Syngamus* sp. ova in wild passerines inhabiting these plantations (Hernandez-Divers, unpub data). *Dyspharynx* sp. has been reported to cause clinical disease in wild birds and

might be an important pathogen for nestlings (60). At least one study suggests that *Ascaridia galli* infections in non-chicken hosts had been acquired from chickens (19). Areas with large concentrations of fecal material, such as chicken feeding stations and corrals are typical in shade-grown coffee parcels and can provide a focus of fecal contamination for the environment and for intermediate hosts (25).

The diseases for which these animals were tested also have significant economic importance for the poultry industry, a rising industry in Costa Rica (62). There is one large commercial poultry operation approximately 20 km from San Luis. Although biosecurity is stringent in these operations, it is important for the Costa Rican authorities to be aware of the diseases free-roaming chickens harbor, in case of a biosecurity breach which might lead to an epizootic.

The addition of chickens to the Monteverde landscape is likely to have an affect on environmental bacterial populations. In particular, we were concerned that an antibiotic resistance plasmids carried by chicken phenotypes would be available for horizontal transfer, making shade-coffee plantations foci for exchange of bacterial genetic material. The poultry literature reports antimicrobial resistance patterns for commercial operations, and within the context of antimicrobial use (2, 7, 14, 66). With the exception of sporadic, individual-animal use of oxytetracycline by three owners, the chickens in San Luis are not routinely exposed to antimicrobials and we did not find a significant difference in the prevalence of tetracycline resistance among those flocks which had been exposed to oxytetracycline and those that were not. Even though this study supports that isolates from free-range chickens display a lower tetracycline resistance than commercial operations, the resistance is still significant (2, 12, 66). This is supported by the presence

of resistance genes *tet(A)* and *te(B).* A variety of genes have been found to mediate resistance to tetracycline; however, *tet(A)-tet(E)* are the most prevalent elements found in tetracycline-resistant *E. coli* isolates, and within that group, the majority of resistance appears to be derived from *tet(A)* and *tet(B)* (55). Current reports in the literature describe higher prevalence of antimicrobial resistance in isolates from wild birds associated with human-disturbed habitats, than of birds that are not exposed to human-associated activities. For example, *E. coli* isolates from black-headed gulls (*Larus ridibundus*) nesting in agricultural regions of the Czech republic displayed a 19% resistance to tetracycline, whereas only 7.6% of *E. coli* isolates from rooks (*Corvus frugilegus*) nesting in remote regions were resistant to tetracycline (15, 49). A recent study of the antimicrobial resistance of *E. coli* isolated from wild birds in the Artic reported low prevalence (8%), but proposes migratory birds as vehicles for transport of resistance genes (64). Class I Integrons, contained within mobile DNA elements, have been shown to be of importance in the transmission of antibiotic resistance in chickens and a useful tool to study antimicrobial resistance transmission (29, 47). The prevalence of *IntI* in our isolates was much lower than previously reported in chickens (29). In addition, no resistance gene cassettes were found, by PCR, integrated in the few integrase positive isolates. Thus Class 1 integron does not appear to play a significant role in the resistance we observed, but remains a potential vehicle of resistance transmission of antimicrobial resistance. In our study, either *tet(A)* or *tet(B)*, but not both, were likely responsible for the recorded tetracycline resistance in the *E. coli* isolates. The results we obtained do not support the theory that antimicrobial use is the primarily selection mechanisms responsible for resistance, but seem to demonstrate that there is a pool of

resistance genes in this population, not necessarily selected through direct antimicrobial use, either therapeutically, or sub- therapeutically in the feed or water. This pool of resistance genes not only poses a threat to the wild bird population but it can be used as a model of microbial transmission within habitats, as has been done in previous reports. (28).

Forest surrogate environments, such as shade-grown coffee plantations provide suitable habitat, which maintains the species richness and abundance of wild birds. However, they are human altered systems which may pose a potential risk to wild birds through exposure to highly mobile backyard chickens and their pathogens. In accord with Friend et al, we recognize the need to focus more attention to disease issues as direct and indirect causes of declining avian populations, from an ecological perspective (23). Unfortunately, disease investigations in wild birds are often only undertaken following a massive mortality event, or on highly endangered species and they typically focus on mortality alone, ignoring subtle, sublethal or indirect effects caused by one, or a combination of diseases. The prevalence and diversity of pathogens of the wild birds sympatric with backyard chickens is currently being investigated (Hernandez-Divers, unpublished data). Although, to our knowledge, no visible mortality events of wild birds has occurred as a result of the introduction of backyard chickens in forest surrogate habitats, we suggest that if wild birds become infected with the aforementioned diseases, there are likely fitness trade-offs to individuals associated with infection and immune defense against viruses and parasite loads, which may translate to effects on population dynamics through indirect and sublethal effects (45). Additionally, free range chickens can serve as reservoirs of antimicrobial resistance bacteria, which could be disseminated

to birds utilizing shade coffee plantations. Recognizing the need for creating more available habitat for avian conservation, further sustainable agroforestry incentives, such as shade-grown cacao, are the wave of the future (21, 25). Thus, studies understanding the disease dynamics of wild birds inhabiting these forest surrogate habitats to determine their significance as foci of risk for diseases will motivate policy changes for conservation organizations.

Table 1. Genotypic antimicrobial resistance for *Enterobacteriacae* isolated from fecal samples was determined from backyard chickens in Costa Rica. Genotypic antimicrobial resistance was determined by testing for *int1*, *tetA* and *tetB* (Integrated DNA Technologies, Coralville, IA). The primer sequences utilized are listed herein.

Gene	Forward primer	Reverse primer	Reference
int1	5'-CCT CCC GCA CGA TGA-3'	5'-TCC ACG CAT CGT CAG GC-3'	3, 29
T*et(A)*	5'-GCT ACA TCC TGC TTG CCT TC-3'	5'-CAT AGA TCG CCG TGA AGA GC-3'	5, 56
Tet(B)	5'-TTG GTT AGG GGC AAG TTT TG-3'	5'-GTA ATG GGC CAA TAA CAC CG-3''	56

Table 2. ELISA antibody titers for Newcastle Disease virus (NDV), infectious laryngotracheitis virus (ILT), infectious bronchitis virus (IBV) and avian pneumovirus (APV) from backyard chickens in Costa Rica.

	No. of samples with titer				
Virus	<1,000	1,000-2,000	2,000-4,000	> 4,000	Total No. tested
NDV	99	19	19	14	151
IBV	95	11	13	9	128
ILT	25	38	15	12	90
APV	39	44	6	1	90

Table 3. ELISA antibody titers for *Mycoplasma gallisepticum* (MG), and *M. synoviae* (MS) from backyard chickens in Costa Rica.

	No. of samples with titer				
Mycoplasma sp.	<1,000	1,000-1,999	2,000-3,999	> 4,000	Total No. tested
MG	95	9	14	29	147
MS	48	8	14	58	128

Table 4. The antibiotic susceptibility pattern, as determined by MIC, of 48 isolates of commensal fecal *Enterobacteriacae* isolates from 13 flocks of backyard chickens in Costa Rica.

	No. of resistant strains/(% strains resistant)[a]						
Organism	TIL	TET	AMP	A/C	TIC	CELOT	GENT
E. coli (n=44)	35/(80)	18/(41)	13/(30)	11/(25)	7/(16)	12/(27)	0
Other *Enterobacteriacae* (n=4)	1/(25)	0	2/(50)	0	1/(25)	0	0

[a] Abbreviations: TIL, tilmicosin; TET, tetracycline; AMP, ampicillin; A/C, amoxicilling with clavulanic acid; TIC, ticarcillin; CELOT, cephalothin; GENT, gentamicin; All isolates were susceptible to florfenicol, difloxacin, ceftiofur, enrofloxacin and orbifloxacin and thus they are not represented on this table. Breakpoints for resistance were determined as per CLSI, 2006 (9).

Table 5. Presence of class 1 integrase and genes associated with tetracycline resistance of 48 isolates from fecal samples of backyard chickens in Costa Rica.

	No. of strains positive/ (% strains positive)			
Organism	*int1*	T*et(A)*	*Tet(B)*	*Tet(A)* and *tet(B)*
All *E. coli* (n=44)	9(20)*	21(48)	4(9)	3(7)
E. coli resistant to tetracycline (n=18)	4(22)*	12(67)	5(28)	3(17)
Other *Enterobacteriacae* (n=4)	2(50)*	0	0	0

*Polymerase chain-reaction of the 5'-3' region failed to show the presence of integrated resistance genes.

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CHAPTER 5

IDENTIFICATION AND ANTIMICROBIAL RESISTANCE PROFILES OF THE FAMILY ENTEROBACTERIACEAE FROM FECAL ISOLATES OF NEOTROPICAL BIRDS

INTRODUCTION

Currently both the general public and medical specialists are concerned about the alarming increase in bacteria that are resistant to antimicrobials (1, 13, 15). Specifically, antibiotic-resistant *Escherichia coli* and several Enterobacteriaceae have been described in domestic poultry, cattle, swine and humans (3, 6, 16). Some reports have speculated that humans (and associated environments, such as hospitals) and intensively-reared domestic animals (and their environments, such as feedlots) may be sources of resistant bacteria which contaminate the environment. These resistant strains could then provide genetic material that confers antimicrobial resistance to other microorganisms, which may, in turn, colonize wildlife populations (10, 30). In fact, a series of publications relating the antimicrobial resistance patterns of wild birds to contact with human or domestic animal waste has recently surfaced (8, 12, 19). Wild bird populations can be vectors of pathogenic *E. coli* and other Enterobacteriaceae. In particular, waterfowl may contaminate surface water destined for human use with a variety of enteric pathogens (10, 18, 29, 31).

Current reports in the literature describe higher prevalence of antimicrobial resistance in isolates from wild birds associated with human-disturbed habitats, than of birds that are not exposed to human-associated activities. For example, *E. coli* isolates from black-headed gulls (*Larus ridibundus*) nesting in agricultural regions of the Czech republic displayed a 19% resistance to tetracycline, whereas only 7.6% of *E. coli* isolates from rooks (*Corvus frugilegus*) nesting in remote regions displayed tetracycline resistance (12, 19). A recent study of the antimicrobial resistance of *E. coli* isolated from wild birds in the Artic reported low prevalence of resistance (8%), but theorized that migratory birds traveling to and from this region might be responsible for transport of resistance genes identified (32).

If indeed it is true that birds inhabiting human-impacted habitats are exposed to and colonized by bacteria which display levels of resistance that are both higher in prevalence and diversity, then this theory can be tested. In Costa Rica, there are similar ecosystems that support comparable avian communities with varied gradients of human activity, from traditional agriculture to relatively untouched forest, which allow for such investigations. We hypothesized that isolates from wild birds living in human-altered landscapes, such as traditional agricultural land, would display higher prevalence and diversity of resistance than those living in forest fragments less affected by humans. In a previous publication, we investigated the genotypic and phenotypic antimicrobial resistance pattern (AMRP) of the free-roaming chickens of San Luis, in the Guanacaste province of Costa Rica and we will use those results for baseline comparisons herein (17). Whereas in the poultry literature, antimicrobial resistance patterns are typically described for commercial operations, and are often discussed in the context of

antimicrobial use, Hernandez-Divers et al were particularly interested in the AMRP of backyard chickens in a region where antibiotic use is relatively rare and where antimicrobials are not a component of the food or water they consume (4, 11). Information on the AMRP of wild birds is still relatively rare and often applied to specific regions and circumstances. We believe this report has a wide application for establishing the background AMRP of tropical avian communities.

MATERIALS AND METHODS

Project Design and Study Sites

Birds were captured in 23 sites which represented 3 levels of human persistence. Human persistence, in this case, is defined as the alteration of forest structure from its "natural" form, the presence of humans, and a measure of human activity such as removal of trees for planting crops or developing pasture; or building dwellings for humans and its associated sewage and waste. The three levels of persistence were represented as follows: 1) No human presence (NHP): secondary old-growth forest with no record of human disturbance to forest structure for at least 50 years, no current human habitation, and little human activity 2) Moderate human presence (MHP): sustainable agriculture such as shade-grown coffee parcels with >40% canopy cover, and which are considered floristically and structurally diverse, thus, less human-associated disturbance to forest structure; little or no human habitation, and little human activity, and 3) persistent human presence (PHP): traditional agriculture such as sun-grown coffee with approximately 7-30% canopy cover with concurrent human habitation and persistent human activity. Six sites were located in the San Luis and 16 in the Las Cruces regions.

San Luis-Six field sites, approximately 1.5 ha each, were chosen in the San Luis valley, located in the Northwest region of Costa Rica in the Monteverde region, in the province of Guanacaste. The San Luis valley is considered a tropical pre-montane zone. Three sites were forest fragments of secondary growth (~75 yrs old), were embedded within larger forest tracks and were categorized as areas of low human disturbance and no permanent human presence (NHP). Three sites were shade-grown coffee plantations and categorized as low human disturbance and minimal human presence (MHP).

Las Cruces-Seventeen sites of 1.5 ha each were chosen in the area surrounding the Las Cruces Biological Station, located near the town of San Vito, within the Coto Brus province, in the Southwest region of Costa Rica. The Las Cruces forest is classified as tropical pre-montane wet forest. The sites considered NHP were embedded in protected forest within the Las Cruces Biological Reserve, or nearby secondary forest (> 50 yrs without disturbance; n=10). Sites of MHP (n=4) were shade-grown coffee plantations as above. Sites of high human activity (PHP; n=3) were sun-grown coffee plantations.

Phenotypic Antimicrobial resistance patterns of fecal flora

Birds were captured with mist nets and individually placed in paper bags until they defecated. Fresh fecal samples were collected from the center of the excrement mound with culturette swabs (Becton Dickinson BBL Culture Swab with Aimes media, BD, Franklin Lakes, NJ), maintained in an ice chest with frozen gel packs and transported back to a laboratory for bacterial processing within 8 hrs of collection. Fecal bacteria was propagated on MacConkey media plates, incubated at 37° C for 12-18 hrs. Growth on enteric media resulted from approximately 40% of the samples obtained. Individual colonies were aseptically collected, introduced into stab storage media

(tryptone 0.2 %, yeast extract 0.02 %, and agar 0.5 % in distilled water) and maintained at 4° C until export. All isolates were imported under appropriate export permits granted by the Costa Rican Ministry of the Environment (MINAE) and imported under a USDA import permit for wildlife products. Once in the USA, bacteria was streaked for isolation on both blood and MacConkey agar plates and incubated at 35±2° C for 12-18 hrs to confirm purity. As we were interested in members of the Enterobacteriaceae family, only lactose fermenters, which made up 55% of the isolates, were selected for further processing. Bacterial isolates were then stored in freezer media (1% peptone, 15% glycerol in distilled water), and frozen at -80° C until further processing. Bacterial identification was done with commercially available Enterobacteriaceae identification strips (API 20E; bioMerieux USA, Durham, NC). The Minimum inhibitory Concentration (MIC), defined as the lowest antibiotic concentration to impede growth of a bacterial colony was determined using commercially available MIC plates following the manufacturer's instructions and previously described standards (9). MIC plates contain a series of titrations for a variety of antibiotics (CMV1PDU and COMEQ2F plates; Trek Diagnostics, Cleveland, OH). The plates were incubated at 37° C for 12 hrs, at which time they were visually inspected for growth. The plate cells which displayed growth were recorded and the first cell without growth was deemed the MIC endpoint. Resistance breakpoints, the antibiotic concentration at or above which a bacteria that displays growth is considered resistant, were determined based on previously published data (9, 25). Table 1a and b illustrates the names and concentrations of the MIC plates utilized. Due to plate availability, two MIC plates were used in this study. The first 71 isolates were realized with the CMV1PDU plate and the following 226 with the

COMEQ2F. Although antibiotics overlapped (n=10), some antibiotics present in CMV1PDU were not present in COMEQ2F and vice versa. As COMEQ2F are commercially available plates, they contained some antibiotics that are routinely ineffective against both gram positive and gram negative bacteria.

Genotypic Antimicrobial resistance patterns of fecal flora.

To determine the presence of Class I Integron gene cassettes (*IntI1*), *tetA* and *tetB*, whole cell templates were made in a standard manner from pure culture stock. Briefly, isolates were plated on blood agar and after 7 hrs of incubation at 37º C, all growth was collected and incubated in 2 ml of BHI (Brain Heart Infusion) broth for 12 hours at 37ºC while stirring. Each tube was vortexed and 1.5 ml of the culture was transferred to a microcentrifuge tube where it was centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the pellet resuspended in 1 ml of 100% ethanol and vortexed again thoroughly. The tube was allowed to incubate at room temperature for 10 min and again centrifuged at 7000 rpm for 10 min. Once again the supernatant was discarded and the pellet resuspended in 1 ml of 1X PBS (Phosphate Buffered Saline) and vortexed thoroughly. It was incubated again at room temperature for 10 min and centrifuged at 7000 rpm for 10 min. The PBS was decanted and the pellet suspended in 1 ml of sterile, deionized water and vortexed thoroughly, incubating it at room temperature for 10 min prior to freezing in -20° C for long term storage until further processing. Polymerase chain reaction was used to identify the samples that contained drug resistance genes such as Class I Integron (*intI1*), and *tetA* and *tetB* as previously described (5, 14, 26). A list of primers utilized is summarized on Table 2. The PCR positive control used was

Salmonella enterica serotype *Typhimurium* DT104 and the negative control was sterile, DNA-free water.

Statistical analysis

A frequency distribution of the 297 isolates was first performed for bacterial genera, considering *E. coli* (E), *Citrobacter* (C), *Klebsiella* (K) or "other Enterobacteriaceae" (O); three levels of Human Persistence (NHP, MHP AND PHP) and Geographic location, San Luis (SL) or Las Cruces (LC). Since we were interested in whether the foraging guild of the bird was associated with the level of resistance, and assuming one mechanism by which birds may be exposed to bacteria that confers resistance is through ingestion of soil, water, or food that contains antimicrobial resistant bacteria, we divided our birds into ground foragers vs. non-ground foragers. Thus, our fourth variable became Ground and Not-Ground. Contingency tables for Genera*Disturbance, Genera*Geographic location and Human Disturbance*Geographic Location showed that bacterial genera are fairly independent of levels of HP, but that HP and Geographic location are highly associated. Therefore, for the purpose of this study, HP was more informative than Geographic location, HP was used in subsequent models. In every case we utilized logistic regression analyses (SAS 9.1, SAS Institute, NC).

We performed 2 types of analyses. Firstly, we were interested in the resistance against each antibiotic separately. Combining the contents of the two MIC plates, we analyzed all of the 26 antibiotics to see how much variability in the resistance rate could be attributed statistically to variation in the four levels of bacterial genera and/or the three levels of Human Persistence. However, when the antibiotic's probability of resistance was <0.05 (close to 0) or > 0.95 (close to 1), differences could not be detected and were

not considered useful. That immediately eliminated those antibiotics to which Enterobacteriaceae are inherently resistant. Therefore, we chose 11 candidate antibiotics with moderate probability of resistance (5%-92%) for further analysis. Two of those 11 were only tested against 71 isolates and those isolates did not adequately represent all HD categories, thus we proceeded with 9 antibiotics of "interest" (ticarcillin, ampicillin, amoxicillin with clauvulinic acid, cephalothin, trimethoprim sulfa, tetracycline, rifampin, cefazolin and cefoxitin).

The second type of analysis aimed at comparing an isolate's measure of multiple resistance. To analyze multiple resistance, a multiple antimicrobial resistance (MAR) score could have been calculated for each isolate by finding the proportion of antibiotics to which isolates were resistant, as has been previously described (23). However, since 226 of the isolates were exposed to one set of 22 antibiotics and the other 71 isolates were exposed to a second set of 14 antibiotics (some which overlapped), this comparison would be flawed. Thus, we first analyzed the 10 antibiotics (see Table 4) which were on both MIC plates for all 297 isolates. For each of the 297 isolates, the proportion of these 10 antibiotics for which isolates were resistant became the response variable, levels of Genera, Human Development, Ground/Not Ground foraging guild, and their interactions as potential explanatory variables. Since there was no statistical difference, we found we could reduce Genera to two levels (*E. coli* and 'Not *E. coli*', collapsing C, K and O) and HP to two levels ('PHP' and 'Not PHP', collapsing MHP and NHP together).

As previously mentioned, the probability of resistance (or proportion) for some of the antibiotics was <0.05 or >0.95 and thus differences by site or genera could not bet detected, thus, we repeated the analysis again, on the 9 previously chosen "antibiotics of

interest: but since some of them were not tested against all 297, we used 226 isolates. This analysis was considered superior. Although we "lost" some isolates in this second analysis, we benefited by comparing antibiotics which displayed moderate levels of resistance and which could best illustrate differences.

RESULTS

Identification of bacterial isolates

Of the 975 wild birds captured in all sites, representing 66 species, fecal samples were obtained from 653. Of those samples, we obtained 297 Enterobacteriaceae isolates, from 44 species of birds. The majority of birds belonged to the Order Passeriforme. Twelve species of birds (27%; most warblers, the Baltimore oriole and the woodthrushes) are North American migrants. Table 3 represents the number isolates obtained from each species, divided into biological families. We further identified the Entobacteriacae isolates as *E. coli* (n=160); *Citrobacter* sp. (n=40); *Klebsiella* sp. (n=26); *Raoultella* sp. (n=17); *Enterobacter* sp. (n=16); *Kluyvera* sp. (n=16); *Serratia* sp. (n=8); *Pantoea* sp. (n=7); and other miscellaneous Enterobacteriaceae genera (n=7).

Phenotypic Resistance

When each antibiotic was analyzed separately, 8 of the 9 (ticarcillin, ampicillin, amoxicillin with clauvulinic acid, cephalothin, trimethoprim sulfa, cefazolin and cefoxotin) a significant difference ($p< 0.05$) was noted for either Genera or HP, and only ticarcillin for both. If significance was found for Genera, the general pattern was that *E. coli* always exhibited less resistance than C, K, or O; and if significance was found for HP, then PHP was always greater than MHP or NHP (Table 4).

Considering multiple resistance, as defined, logistic regression showed that Genera had only 2 significant levels: 'E. coli' and 'Not E. coli' (or 'OKC'), while HP also had only two significant levels: 'PHP' and 'Not PHP' (which is NHP, and MHP combined). For the analysis of 10 antibiotics against 297 isolates, and including Ground vs. NotGround, there appeared to be a significant difference for Ground; however, the log-odds (logit) regression coefficient (estimate) is negative, which indicates the significance is not valid (Appedix 1a). Thus, we did not include foraging guild in the subsequent analysis (Appendix 1b). As mentioned, we repeated the analysis for the 9 antibiotics of interest against 226 isolates and found, as with 10 antibiotics, a significant difference explained by level of HP (Appendix 1c).

Genotypic resistance

Of the 297 isolates tested for genes associated with resistance in chickens, only 2 (0.7%) contained *intI1* and one (0.3%) contained *tetB*. Table 4 represents the distribution of the 37 (12%) isolates found to contain *tetA*.

DISCUSSION

Identification of bacterial isolates

Fecal samples were collected from approximately 67% of birds captured. Birds often defecate immediately upon entanglement in mist nets and thus smaller birds with very fast metabolisms may not be as likely to produce a second fecal sample for collection. Approximately 45% of the fecal samples collected yielded growth of enteric organisms. One of the factors affecting recovery of fecal bacteria is initial fecal sample size; therefore, larger birds from

which we collected larger samples (>40 g) are overrepresented. The diet of a bird dictates its gastrointestinal flora, and thus, insectivorous and omnivorous birds, when compared with strict frugivores, are more likely to yield Gram negative fecal isolates. In fact, less than 2% of birds captured are considered strict frugivores and isolates from those represent less than 1.6% of the total number of isolates. More than half (54%) of all fecal isolates were *E. coli* but several common Enterobacteriaceae genera were represented. Middleton et al. recovered *E. coli* from Canada geese fecal samples at rates that varied from 56-100%, depending on the sampling season (22). We would expect *E. coli* to make up a higher proportion of the normal fecal flora of Canada geese due to their size, diet and foraging behavior. To our knowledge, this is the first report of the specific identity of fecal Gram negative isolates from a neotropical Passerine community. These results, in combination with the antibiotic resistance profiling of this report maybe useful in future studies that aim to understand non-point sources of fecal contamination through microbial source tracking (23).

Phenotypic resistance

Of the antibiotics tested, resistance to clindamycin, penicillin, erythromycin, oxacillin and rifampin was very high, regardless of genera, or degree of human persistence. This level of resistance against these antibiotics was expected, as these antibiotics were primarily designed to inhibit the growth of Gram positive bacteria and thus members of the Enterobacteriaceae are inherently resistant (28). Except for rifampin, the resistance against those

antibiotics was > 95% and, as explained before, they were eliminated from the analysis. Complete susceptibility to ceftiofur, gentamicin, cefpodoxime, imipenem and amikacin was also almost 100% and is consistent with *in vitro* trials with these antibiotics against Enterobacteriaceae (28). Once again, those antibiotics were not part of the statistical analysis. It is interesting to note that this pattern of resistance/susceptibility was conserved regardless of the differences in species of the two avian communities and despite two similar, yet distant, geographic locations. This suggests that fecal flora and its inherent resistance pattern is at least comparable across bird families and maintained across regions.

Conversely, significant differences in resistance patterns occurred for ticarcillin, ampicillin, amoxicillin with clauvulinic acid, cephalothin, trimethoprim sulfa, cefazolin and cefoxotin. To our knowledge, there is only one similar study reporting on the antimicrobial resistance of similar Passerines from the Brazilian Atlantic forest. In that study they analyzed 191 isolates from 19 birds and found much higher levels of resistance against ampicillin (57%), chloremphenicol (35%) and tetracycline (11%). However, the specific identity of the isolates was not reported and therefore comparisons with this study are not possible (24).

In our study, and discounting antibiotics to which Enterobacteriaceae is inherently resistant, multiple antibiotic resistance was less common than other alternative profiles. Less than half (41%) of the isolates exhibited complete susceptibility to all antibiotics, 19% were resistant to at least one antibiotic, and

25% of the isolates exhibited resistance to three or more antibiotics. For clarity, the following tables represent the logit terms and probability of resistance such that:

	(MHP, NHP)	**PHP**
E. coli	-3.39	-2.91
(C, K, O)	-1.26	-0.78

	(MHP, NHP)	**PHP**
E. coli	6%	9%
(C, K, O)	21%	27%

Thus, isolates from the 'CKO' group displayed significantly higher probability of being resistant than those from the '*E. coli*' group, 21% vs. 6%, respectively, in Low or Medium Human Persistence locations. Isolates from the 'High HP' level were statistically significantly more resistant than those from the 'NHP' or 'MHP' level, but the magnitude of the difference was smaller - an average increase from 6% resistance to 9% resistance for '*E. coli*' isolates and an average increase from 21% to 27% resistance for 'CKO' isolates. Finally, foraging guild (Ground/NotGround) was not particularly informative in explaining resistance rate, especially after controlling for Human Disturbance and Genera type. These results support our expectations that bacterial genera has a strong influence on the level and type of antibiotic resistance and that the isolates from the sites with the highest degree of human disturbance displayed significantly higher probability of resistance.

Interestingly, the fact that our *E. coli* isolates displayed lower resistance level than other Enterobacteriaceae is counterintuitive, as *E. coli* has been found to have a comparatively rapid mutation rate. In the presence of an antibiotic, a rapid mutation rate might be beneficial for natural selection, and allow *E. coli* to remain ahead of the antimicrobial arms race better, when compared to other genera in Enterobacteriaceae (27). However, the distinction might lie in subtle strain differences which were not investigated in this study. We did not find a significant difference in antimicrobial resistance when we analyzed birds by foraging location (Ground vs. NotGround). This is not surprising, as, except for a small minority of species which absolutely spend the majority of their time on the ground, most of the species captured likely utilize a variety of forest strata. Capturing birds via mist nets translates to a capture community composed of birds that primarily utilize the understory and excludes canopy birds, which might have exhibited a significantly different pattern of resistance.

There are several explanations for antimicrobial drug resistance development in fecal bacteria of neotropical birds in the absence of documented exposure to antimicrobials. First, resistance can develop through spontaneous mutation, or it can be acquired by horizontal gene transfer from other microbes via conjugation. During conjugation, plasmids in one organism that are responsible for resistance to antibiotics may be transferred to an organism that previously did not possess such resistance. Lastly, bacteria can incorporate into their own genetic machinery foreign pieces of DNA by either of two types of

DNA transposition. In transformation, DNA from the environment (e.g. from the death of other bacteria) is absorbed into the bacterial cell and in transduction, a piece of DNA is transported into the cell by a virus. Thus, we concede that antimicrobial resistance can develop in the absence of human-related activities. However, it is clear from our results that resistance was more significant in areas of high human persistence. Although it is difficult to measure the degree of human activity in the different sites, sites categorized as HHP had more frequent human habitation, and a greater abundance and diversity of domestic animals (e.g. pigs, livestock, and other domestic birds in addition to chickens, such as waterfowl), and thus the degree of human and domestic animal waste that might contain bacterial strains or resistance genes available for exchange was likely higher (20, 21). Additionally, the make up of the avian community sampled might explain the patterns of resistance, since 25% of the species sampled in HHP sites are "highly associated with human-inhabited areas" as compared to 12% of those in LHP and MHP combined (34).

Interestingly, there was no statistical difference in the resistance frequency or prevalence of isolates from birds in sustainable agricultural sites (shade coffee plantations) and forest sites, suggesting that the management of these plantations maintains the integrity of forested habitat in ways not previously explored.

Genotypic resistance

Integrons have been identified in mobile DNA elements that allow for the transfer of antimicrobial resistance. In particular, Class I Integron has been shown to contain one

or more genetic elements that encode for antibiotic resistance in chickens (2, 14). The prevalence of *intI1* in these wild bird isolates was much lower than previously reported in chickens in the San Luis region (14, 17). In addition, no resistance gene cassettes were found, by 5'-3' PCR detection, integrated in the few integrase positive isolates. Thus Class 1 integron appears to not play an insignificant role in the resistance we observed, but remains a potential vehicle of resistance transmission of antimicrobial resistance. Unfortunately, testing of other genes thought to be responsible for conferring resistance against the antibiotics of interest was not possible but planned for the future. We were interested, however, in the presence of genes conferring resistance to tetracycline, as we had found this to be prevalent in the chickens previously surveyed (17). In addition, *tet* genes were of interest because oxytetracycline was the only antibiotic reported to be used (albeit in small quantities) in chickens. A variety of genes have been found to mediate resistance to tetracycline; however, *tetA*-*tetE* are the most prevalent elements found in tetracycline-resistant *E. coli* isolates, and within that group, the majority of resistance appears to be derived from *tetA* and *tetB* (33). In this report, *tetA* was likely responsible for the recorded tetracycline resistance.

Phenotypic and genotypic antimicrobial resistance is a complex issue that is affected by a variety of factors, including species, health status of individual, age, diet, animal production type, bacterial strain, sample and laboratory methodology, geographic location, antimicrobial use, etc. However, the results we obtained do suggest that human persistence (and its associated activities) in these habitats is positively related to higher prevalence of antimicrobial resistance in wild birds that share these habitats.

Table 1a. The CMVIPDU plate (Trek Diagnostics, Cleveland, OH) was used on the first 71 isolates analyzed. The layout of the plate, including antibiotic names and concentrations (μg/ml), is illustrated herein.

Ticar 2	Ticar 4	Ticar 8	Ticar 16	Ticar 32	Ticar 64	Ticar 128	Spect 8	Spect 16	Spect 32	Spect 64	Spect 128
Amp 0.06	Amp 0.125	Amp 0.25	Amp 0.5	Amp 1	Amp 2	Amp 4	Amp 8	Amp 16	Amp 32	A/C 1/0.5	A/C 2/1
A/C 4/2	A/C 8/4	A/C 16/8	A/C 32/16	Celot 1	Celot 2	Celot 4	Celot 8	Celot 16	Celot 32	Ceftio 0.06	Ceftio 0.125
Ceftio 0.25	Ceftio 0.5	Ceftio 1	Ceftio 2	Ceftio 4	TMS 0.5/9.5	TMS 1/19	TMS 2/38	TMS 4/76	Diflox 0.12	Diflox 0.25	Diflox 0.5
Diflox 1	Diflox 2	Diflox 4	Diflox 8	Tilm 4	Tilm 8	Tilm 16	Tilm 32	Tilm 64	Enro 0.03	Enro 0.06	Enro 0.125
Enro 0.25	Enro 0.5	Enro 1	Enro 2	Enro 4	Florfe 0.25	Florfe 0.5	Florfe 1	Florfe 2	Florfe 4	Florfe 8	Genta 0.12
Genta 0.25	Genta 0.5	Genta 1	Genta 2	Genta 4	Genta 8	Genta 16	Orbifl 0.12	Orbifl 0.25	Orbifl 0.5	Orbifl 1	Orbifl 2
Orbifl 4	Orbifl 8	Tet 0.25	Tet 0.5	Tet 1	Tet 2	Tet 4	Tet 8	Tet 16	POS	POS	POS

*Abbreviations: Ticar= Ticarcillin; Spect=Spectinomycin; Amp=Ampicillin; A/C=Amoxicillin with clauvulinic acid; Celot=Cephalothin; Ceftio=Ceftiofur; TMS=trimethoprim sulfamethoxazole; Diflox=Difloxicin; Tilm=Tilmicosin; Enro=Enrofloxacin; Genta=Gentamicin; Orbifl=Orbifloxicin; Tet=Tetracycline; POS=Positive Control.

Table 1b. The contents of the COMEQ2F plate, which was used for the remaining 226 isolates, is illustrated herein.

TMS 2/38	TMS 1/19	TMS 0.5/9.5	Amp 16	Pen 8	Celot 16	Genta 8	Enro 4	Tet 8	Chl 16	Fox 16	Ery 4
Ami 32	A/C 32/16	Tim 64/2	Amp 8	Pen 4	Celot 8	Genta 4	Enro 2	Tet 4	Chl 8	Fox 8	Ery 2
Ami 16	A/C 16/8	Tim 32/2	Amp 4	Pen 2	Celot 4	Genta 2	Enro 1	Tet 2	Chl 4	Fox 4	Ery 1
Ami 8	A/C 8/4	Tim 16/2	Amp 2	Pen 1	Celot 2	Genta 1	Enro 0.5	Tet 1	Ceftio 4	Fox 2	Ery 0.5
Ami 4	A/C 4/2	Tim 8/2	Amp 1	Pen 0.5	Faz 16	Pod 16	Ticar 64	Mar 2	Ceftio 2	Imi 8	Ery 0.25
Rif 2	Rif 1	Amp 0.25	Amp 0.5	Pen 0.25	Faz 8	Pod 8	Ticar 32	Mar 1	Ceftio 1	Imi 4	POS
Oxa + 4	Clin 2	Clin 0.5	Clin 0.25	Pen 0.12	Faz 4	Pod 4	Ticar 16	Mar 0.5	Ceftio 0.5	Imi 2	POS
Oxa+ 2	Orbfl 4	Orbfl 2	Orbfl 1	Pen 0.06	Faz 2	Pod 2	Ticar 8	Mar 0.25	Ceftio 0.25	Imi 1	POS

*Abbreviations: TMS=Trimethoprim sulfamethoxazole; Amp= Ampicillin; Pen=Penicillin; Celot=Cephalothin; Genta=Gentamicin; Enro=Enrofloxacin; Tet=Tetracycline; Chl=Chloramphenicol; Fox=Cefoxitin; Ery=Erythromycin; Ami= Amikacin; A/C=Amoxicillin with clavulanic acid 2:1 ratio; Tim=Ticarcillin with clauvulanic acid; Ceftio= Ceftiofur; Pod=Cefpodoxime; Ticar=Ticarcillin; Mar=Marbofloxicin; Imi=Imipenem; Oxa+=Oxacillin with 2% NaCl; Clin=Clindamycin; Orbfl=Orbifloxicin; POS= Positive control.

Table 2. Genotypic antimicrobial resistance was determined by testing for *intI1*, *tetA* and *tetB* (Integrated DNA Technologies, Coralville, IA). The primer sequences utilized are listed herein.

Gene	Forward primer	Reverse primer	Reference
intI1	5'-CCT CCC GCA CGA TGA-3'	5'-TCC ACG CAT CGT CAG GC-3'	(5, 14)
tetA	5'-GCT ACA TCC TGC TTG CCT TC-3'	5'-CAT AGA TCG CCG TGA AGA GC-3'	(7, 26)
tetB	5'-TTG GTT AGG GGC AAG TTT TG-3'	5'-GTA ATG GGC CAA TAA CAC CG-3''	(26)

Table 3. Two hundred and ninety seven Enterobacteriaceae isolates were collected from 44 species of tropical birds from Costa Rica.

Family	Species name	No. of isolates	
		Other Enterobacteriaceae	*E. coli*
Momotidae (Mot mots)	*Momotus momota*	4	4
Parulidae (Warblers)	*Basileuterus rufifrons*	3	5
	Wilsonia canadensis	5	0
	Mniotilta varia	1	0
	Oporornis formosus	2	1
	Vermivora peregrina	0	1
	Wilsonia pusilla	1	1
	Seiurus aurocapillus	3	2
Icteridae (Icterids)	*Icterus g. galbula*	0	1
	Amblycercus holosericeus	1	0
Corvidae (Crows and jays)	*Cyanocorax morio*	1	0
Thraupidae (Tanagers)	*Saltator maximus*	2	5
	Chlorospingus opthalmicus	3	0
	Habia rubica	2	0
	Tiaris olivacea	3	4

	Ramphocelus costaricensis	0	2
Cardinalidae (Cardinal-like birds)	*Piranga rubra*	2	0
Turdidae (Robins and Nightingales)	*Turdus grayi*	17	22
	Catharus aurantiirostris	16	28
	Catharus ustulatus	4	3
	Hylocichla mustelina	3	10
	Turdus assimilis	9	26
	Turdus plebejus	0	1
Troglodytidae (Wrens)	*Troglodytes aedon*	0	1
	Thryothorus rufalbus	16	6
	Thryothorus modestus	5	2
	Henicorhina leucophrys	1	0
Tyrannidae (Flycatchers)	*Mionectes oleagineus*	3	1
	Myiarchus tuberculifer	1	0
	Rhynchocyclus brevirostris	1	0
	Tolmomyias assimilis	1	0
	Contopus sordidulus	1	0
Furnariidae (Woodcreepers)	*Sittasomus griseicapillus*	1	0
	Dendrocincla homochroa	5	1
	Dendrocolaptes certhia	0	1

Vireonidae (Vireos)	*Vireo flavoviridis*	1	0
Emberizidae (Sparrows)	*Melozone leucotis*	9	15
	Arremonops conirostris	0	1
	Arremon aurantiirostris	2	4
Pipridae (Manakins)	*Chiroxiphia linearis*	3	2
Columbidae (Doves)	*Leptotila verreauxi*	1	4
	Leptotila cassini	0	4
Ramphastidae (Ramphastids)	*Aulacorhyncus prasinus*	0	1
Fringillidae (Finches)	*Atlapetes gutturalis*	1	0

Graph 1. The overall percentage of resistance of isolates tested against 26 antibiotics is represented herein. The sample size of isolates is indicated above the columns to clarify against which antibiotics isolates were tested.

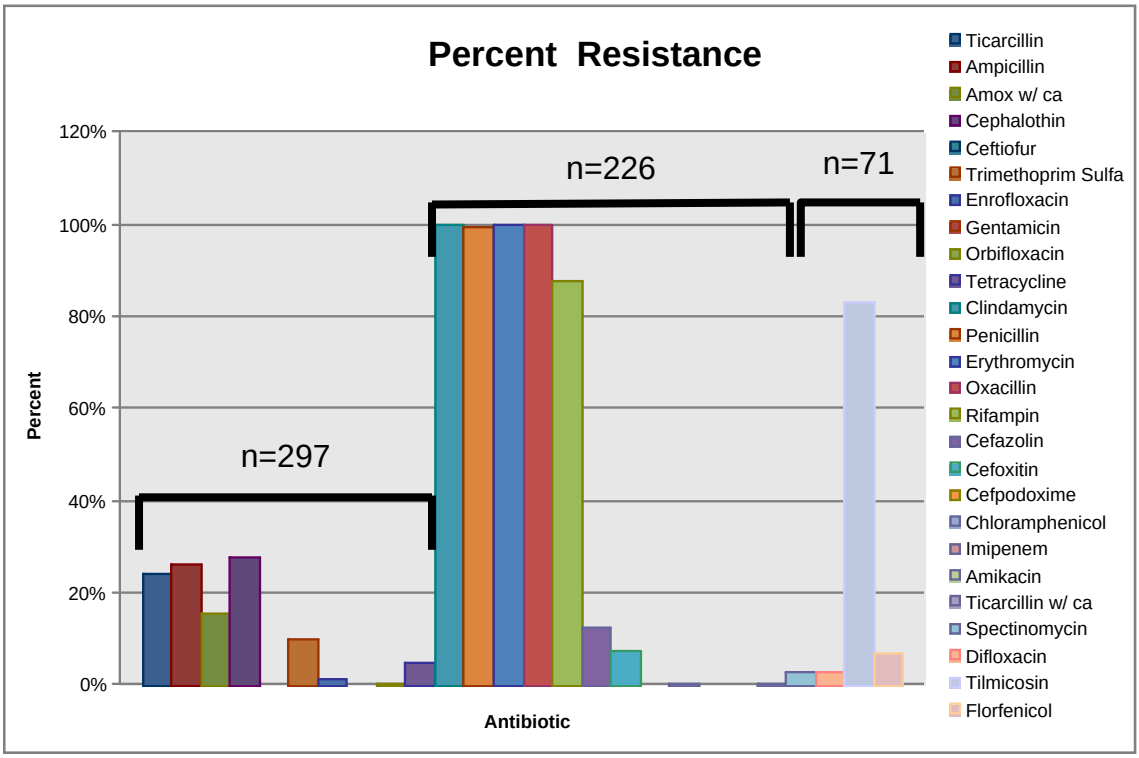

Table 4. Twenty six antibiotics were analyzed for resistance individually. The number of isolates tested against each antibiotic, the number found resistant, the number found to display intermediate resistance, and the proportion of resistance is represented. The proportion of resistance was utilized to analyze the differences in resistance based on either antibiotic Genera, or level of Human Persistence. Significance was determined at 0.05 level. Whenever no significance was found between levels, they were collapsed.

Name of Antibiotic	Total # of Isolates	# Isolates Resistant	# Isolates Intermediate Resistance	Proportion of Resistance	Significance based on Genera	Significance based on HP
Ticarcillin	297	72	9.5	0.2744	E<C<(K,O)	(NHP,MHP)<PHP
Ampicillin		78	12.5	0.3047	E<(C,O)<K	-
Amoxicillin w/ clauvulinic acid		46	18.5	0.2171	(E,K)<(C,O)	-
Cephalothin		82	33.5	0.3888	(E,K)<(C,O)	-
Ceftiofur		0	1	0.0033	-	-
Trimethoprim Sulfa		30	0.5	0.1027	-	MHP<NHP<PHP
Enrofloxacin		4	0	0.0135	-	(NHP, MHP)<PHP
Gentamicin		0	0.5	0.0017	-	-
Orbifloxacin		1	0.5	0.0051	-	-
Tetracycline		14	0	0.0471	-	-
Clindamycin	226	226	0	1	-	-
Penicillin		225	0	0.9956	-	-
Erythromycin		226	0	1	-	-
Oxacillin		226	0	1	-	-
Rifampin		198	9.5	0.9181	-	-
Cefazolin		28	17.0	0.1991	-	NHP<MHP<PHP
Cefoxitin		17	14.0	0.1372	-	MHP<NHP<PHP
Cefpodoxime		0	1	0.0044	-	-
Chloremphenicol		1	0.5	0.0066	-	-
Imipenem		0	0	0	-	-
Amikacin		0	0.5	0.0022	-	-
Ticarcillin w/ clauvulinic acid		1	0.5	0.0066	-	-
Spectinomycin	71	2	32.5	0.4859	-	-
Difloxacin		2	1	0.0422	-	-
Tilmicosin		59	0	0.2611	-	-
Florfenicol		5	22.5	0.3873	-	-

Table 4. The distribution of 37 isolates containing *tetA* is represented.

	E. coli	*Citrobacter*	*Klebsiella*	Other Enterobacteriaceae
PHP	0	0	0	0
MHP	7	0	2	7
NHP	10	3	1	7

Appendix 1a and b. SAS ouput for the logistic regression of 10 antibiotics that were tested against all 297 isolates and considering Ground vs. NotGround as an explanatory variable. Since the logit term was negative, the analysis was repeated and thus 1b reflects that analysis without the foraging location.

Type 3 Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
NG1	1	123.1217	<.0001
HP1	1	4.4480	0.0349
GR	1	3.9801	0.0460

Analysis of Maximum Likelihood Estimates

Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept	1	-2.5545	0.1226	433.9898	<.0001
NG1 CKO	1	1.3475	0.1214	123.1217	<.0001
HP1 HDD	1	0.3559	0.1687	4.4480	0.0349
GR	1	-0.2213	0.1109	3.9801	0.0460

Response Profile

Ordered Value	Binary Outcome	Total Frequency
1	Event	403.5

Ordered Value	Binary Outcome	Total Frequency
2	Nonevent	2566.5

Class Level Information

Class	Value	Design Variables
NG1	E	0
	CKO	1
HD1	PHP	1
	MHPNHP	0

Appendix 1b.

Type 3 Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
NG1	1	130.1962	<.0001
HP1	1	4.1927	0.0406

Analysis of Maximum Likelihood Estimates

Parameter		DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept		1	-2.6930	0.1028	686.6316	<.0001
NG1	CKO	1	1.3761	0.1206	130.1962	<.0001
HP1	PHP	1	0.3447	0.1683	4.1927	0.0406

Appendix 1b. The SAS output of the analysis of 9 antibiotics of interest againsy 226 isolates.

Response Profile

Ordered Value	Binary Outcome	Total Frequency
1	Event	275
2	Nonevent	1759

Class Level Information

Class	Value	Design Variables
NG1	E	0
	OCK	1
HP1	H	1
	MHP LHP	0

Type 3 Analysis of Effects

Effect	DF	Wald Chi-Square	Pr > ChiSq
NG1	1	129.4550	<.0001
HP1	1	6.5373	0.0106

Analysis of Maximum Likelihood Estimates

Parameter		DF	Estimate	Standard Error	Wald Chi-Square	Pr > ChiSq
Intercept		1	-3.3871	0.1746	376.4504	<.0001
ng1	OCK	1	2.1316	0.1873	129.4550	<.0001
HP1	HHP	1	0.4754	0.1859	6.5373	0.0106

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CHAPTER 6

COMPARING PATHOGEN PREVALENCE AND DIVERSITY OF WILD NEOTROPICAL BIRDS IN TWO HABITAT TYPES

INTRODUCTION

The field of free-ranging avian disease investigation has been driven by either highly visible events of mortality, especially in species which are considered a resource (e.g. waterfowl), or by investigations of diseases that could affect humans or domestic animals (e.g. avian influenza or Mycoplasmosis respectively). More recently, the variety of environmental factors that could cause changes in disease dynamics has received more attention and raised new questions about disease dynamics. Chytridiomycosis, a fungal disease of amphibians, exemplifies this phenomenon, in that, after more than a decade of work defining the pathogen-host relationship, certain pieces of the puzzles still do not fit. A variety of explanatory factors such as climate, pollution, or environmental factors causing immunosuppression have thus far failed to definitively explain the emergence, host and geographic distribution of this disease (10, 32, 49).

The emergence of new diseases, new hosts, or new geographic locations, is on the rise, even when controlling for reporting effort. For example, recent examination of the spatial pattern of a variety of categories of emerging diseases found that human population density was a common and significant predictor of emerging disease events, thus supporting the hypothesis that disease emergence is largely a product of anthropogenic and demographic changes (22). Simultaneously, the threat that disease poses for wildlife populations is significant, particularly for those species whose available habitat is shrinking. Therefore, we suggest a more holistic approach into wildlife disease investigation that calls for examining habitat characteristics and anthropogenic factors which may facilitate disease emergence and transmission. Our study, in which we explored the differences in health parameters, pathogen prevalence and diversity of a neotropical bird community in two different habitats shade-grown coffee and natural forest, could serve as an example of this type of approach and highlights the limitations and complexities of such an approach.

A comparison of disease dynamics of wild birds in shade-grown coffee and forest is particularly relevant because the conservation community has identified shade-grown coffee as both a desirable economic model as well as a surrogate for forested habitat. In fact, the literature abounds with examples of the positive effects shade-grown coffee has on avian biodiversity, mostly focusing on diversity indices (33, 36). Species diversity indices, typically used to describe communities in shade coffee, are useful in assessing species richness and relative abundances within and between habitats, but they have limitations as they ignore species composition, ecological functions and fitness (23). More recently, studies investigating the potential ecological costs and benefits of species

using shade-grown coffee versus coffee grown without shade have begun to appear in the literature (11, 38, 45). Our approach, in which we measure both health parameters and the prevalence and diversity of avian pathogens, could help answer a critical question about the costs and benefits of shade-grown coffee for wild birds; namely, do the wild birds that use these habitats show evidence of increased exposure to pathogens and does it matter for individuals or populations, i.e., are they less healthy than conspecifics living in nearby forest habitat? And more generally, does it provide useful information that would allow us to make intelligent predictions about change in disease dynamics in sustainable agricultural land in the tropics?

MATERIALS AND METHODS

Project Design

We captured wild birds in three shade-grown coffee plantations and three forest fragments. To determine capture effort, we recorded the total time nets were open per day, as well as the number of nets. To increase our number of replicates (individual birds), we aimed to capture 30, not previously captured birds, at each site during each sampling bout. Sampling bout was defined as the time spent in Costa Rica capturing birds. From 2005-2008, we realized eight sampling bouts. We aimed for those sampling bouts to be representative of both seasons (wet vs. dry) and avian reproductive activity (breeding vs. non-breeding periods). Table 1 represents the sampling bouts and the relevant seasonal and reproductive levels.

Avian Capture

Birds were captured with mist nets following standard methodology (5). Approximately 8-14 nylon mist nets (38 mm nylon, 4 panels, either 6 or 9 mm in length) were erected the evening before a capture day and maintained furled. The morning of capture, the nets were opened between 5-7 AM, and maintained open until 1-2 PM or until 10 birds not previously banded were captured per day. The nets were monitored every 30 min. Nets were opened on two alternating sites, to avoid capturing in one site on two consecutive days, which leads to a decrease in capture effort. Human activity was not permitted near the nets while they were open, other than to extract birds. The nets were closed early if it rained substantially, or if it was very windy. Wet nylon, in combination with wet feathers, inflicts abrasions and bruising small birds. Additionally, small birds become hypothermic in a short time when captured in a mist net during the rain. Wind renders the mist nets visible (by movement and by debris that becomes lodged on the nets), reducing capture effort. Birds were extracted from the nets and placed in disposable brown paper bags. Time of capture was recorded. Birds were transported to a "base camp" where they were processed. Birds were processed immediately to minimize holding time. In most cases, birds were released within 20 min of extraction from the nets, and released near the point of capture. If a bird suffered an injury that would render it non-releasable, a rare event, it was immediately euthanized by cervical dislocation. All avian capture and handling techniques were reviewed and approved by the University of Georgia's Animal Care and Use Committee. A bird was weighed while still in the bag. Birds were banded on the left leg if utilizing the smallest of band size, and on the right leg if any other size was used. The bands were color coded for the site and consecutively numbered. They were made of ultra-violet sensitive plastic and are expected to degrade

and fall off after 4-5 years. The following morphometric measurements were obtained: body weight, culmen, tarsometatarsal length and width, and wing cord. If sexually dimorphic, gender was noted. If displaying juvenile characteristics, age was determined. Other information noted and recorded included: molting pattern, any physical abnormality, and presence of brood patch or egg in the abdomen. If a bird was recaptured within the same sampling bout, no samples were collected again.

Target Species

Although all birds (except hummingbirds) captured in mist nets were processed, six focal species were selected for further analyses which were otherwise logistically and financially impossible on all birds. The six focal species were selected based on the following criteria: 1) they were among the top ten most frequently captured species in both habitat types, 2) they represented a variety of foraging guilds (from strict frugivory to omnivory), 3) they were permanent residents (e.g. not Neotropical migrants), 4) they represented a variety of foraging positions (which might be related to disease transmission, particularly from backyard chickens which are strict ground foragers) from "ground" to "middle canopy", 5) were large enough for sample collection, and 6) had relatively small home ranges (47). Table 2 represents the names and characteristics of the focal species.

Avian Health Parameters

Three subjective health scores were obtained: 1) body mass, 2) body condition score based on pectoral muscle density, and 3) and ectoparasites score. Body mass was defined as either body weight/tarsus length or body weight/wing cord, which are acceptable methods for standardizing body mass (19). Body condition based on pectoral

muscle density was scored from 1-5/5 such that 1=emaciated, 2=thin, 3=ideal, 4=overweight, 5=obese (42). An ectoparasites score was generated by counting mites on one wing and ranged from 1-5; 1=no mites noted, 2=1-25 mites, 3=25-50, 4=50-100, and 5= >100 mites (19).

Blood Collection

Blood collection was the last procedure performed before returning birds to individual paper bags for release. Venipuncture was achieved from the jugular vein in birds which weighed > 20 g, whereas the superficial ulnar vein was utilized in birds < 20g. Ulnar vein puncture and blood collection with a heparin-lined capillary tube was achieved in birds that weighed $\leq$ 15-10 g. No more than 1% of body weight of blood volume was collected.

Avian enteric parasites

Fecal samples were collected from paper bags and preserved in a 2.5% potassium dichromate solution until examined. Samples were examined directly, by fecal smear, and by standard flotation technique with Sheather's sugar solution and examined microscopically (48).

Avian ectoparasites

Feather mites were collected by sharply dissecting a small window of mite and mite eggs on the wing feathers. Body and feather lice, ticks or parasitic flies were collected directly. All arthropods were stored in 70% ethanol and identified by morphological characteristics.

Avian hemoparasites

A thin blood smear was made immediately after blood collection. The blood smears were dried, stained with Wright's stain, and examined for the presence or absence of hemoparasites in the following manner. The entire slide was scanned at 100 X for the presence of microfilaria or other large hemoparasites and to find the most appropriate area to examine at higher magnification. The slide was then scanned at 1,000 X for at least 10 min to examine for hemoparasites (48). All suspect blood smears with hemoparasites were subsequently reviewed by a clinical pathologist who confirmed the identification.

Avian Serology

Blood tubes collected in the field were maintained in a cooler with ice and centrifuged no more than 8 hrs after collection. Following centrifugation, the serum was transferred to cryovials, and frozen in a -80° C freezer until processing. In order to determine disease seroprevalence, hemoagglutination inhibition was performed for paramyxovirus, and whenever plasma volume allowed, *Mycoplasma gallisepticum*, *M. synoviae* and Infectious Bronchitis Virus (IBV).

***Mycoplasma spp.*nucleic acid detection**

Choanal swabs were collected from the aforementioned target species to determine the presence of *Mycoplasma gallisepticum* and/or *M. synoviae* nucleic acid. The swabs were placed into 1.5 ml microcentrifuge tubes containing 300 µml of sterile PBS. The day of collection, DNA was extracted utilizing a commercial available Qiamp Mini kit (Qiagen, Valencia, CA) following the manufacturer's recommendations. The resultant solution was frozen at -70° C until processing. To determine the presence of

Mycoplasma gallisepticum DNA, a Real-time Taqman® polymerase chain reaction (R-PCR) was utilized as previously described (7). A plasmid containing the amplification target sequence of the MGA0319 gene (lp gene; GenBank Accession # NC_004829) previously described by Callison *et. al*., was utilized as positive control. To determine the presence of *Mycoplasma synoviae* DNA in each sample, a PCR assay was utilized as previously described by Lauerman *et. al* (24). Positive controls utilized were reference strains WVU1853 and F102AS.

Paramyxovirus amplification and isolation.

Cloacal swabs were aseptically collected from target species. The swabs were immersed in sterile microcentrifuge tubes containing Dulbecco's Modification of Eagle's Medium (DMEM; Mediatech, Manassas, VA) cell culture media with added antimicrobials (amphotercin B at 1%, gentamicin it is 0.1% and penicillin/streptinomycin at a concentration of 5000 IUs/ml) and frozen for later processing. To realize virus amplification and isolation, the tubes are vortexed, the swabs were discarded and the tubes centrifuged at 10,000 rpm for 5 min. The supernatant was loaded on to a sterile syringe and injected into ten day old embryonated chicken eggs. After 72 hrs, allantoic fluid was collected and utilized for a hemagglutination assay. One drop of fluid from each isolate was placed on cards designed to inactivate cells, yet capture and preserve nucleic acid, (FTA© classic cards, Whatman International Ltd, Springfield Mill, James Whatman Way, Maidstone, Kent, UK) for storage. Extraction of DNA from the FTA cards for the molecular detection of Newcastle disease virus was performed as previously described (37).

Statistical Analyses

For avian health parameters, a repeated measures model that recognized multiple observations as belonging to the same bird when repeated captures occurred was used to analyze health parameters for differences due to habitat type (coffee vs. forest), season (wet vs. dry) or reproductive season (breeding vs. non-breeding) for each of six target species separately. Habitat type, season, reproductive season and a habitat*season interaction term were included in the model as fixed variables while band ID was included as a random factor. An unstructured covariance structure was used. When repeated measurements on the same bird were not present (TarsusW) then an analysis of variance (ANOVA) was used instead without the bandID factor. In addition, LTM species were tested for gender and age effects and WEGS were tested for age effects using a repeated measures model. Factors of HabitatType and age or gender and a 2-way interaction effect were included in the model. If reproductive season was significant it was also included in the model. All hypothesis tests were 2-sided and the significance level was $\alpha = 0.05$. The analysis was performed using PROC MIXED in SAS V 9.1 (Cary, NC).

For hemoparasites and endoparasites, Chi-square tests were used to test for associations between presence/absence of *Haemoproteus* or microfiliaria, or, in the case of endoparasites, presence/absence of five categories of endoparasites (coccidian, cestode, trematode, acanthocephalan and nematode), and habitat type (coffee, forest), Reproductive Season (Breeding, Non-Breeding), Age (Adult, Juvenile), Presence in Ground (Yes/No), Undergrowth (Yes/No), Middle Canopy (Yes/No) or Upper Canopy (Yes/No) for each breed separately. If there were less than 24 birds in a species than

Fisher's exact test was used instead. There needed to be birds in each of the four boxes of the 2x2 contingency table for tests of association to be performed. When a significant difference between coffee and forest was found for a specific species, Chi-square analysis was used to determine if there was a difference by specific sampling site. A score (0-5) reflecting the different categories of parasites infecting an individual bird was created to illustrate the "diversity" of parasite fauna. A repeated measures model was used to test the total number of endoparasite infections for differences due to habitat type (forest vs. coffee), season (wet vs. dry) or reproductive season (breeding vs. non-breeding) for all species pooled. Habitat type, season, reproductive season and a habitat*season interaction term were included in the model as fixed variables while band ID was included as a random factor. An unstructured covariance structure was used.

RESULTS

Avian Health Parameters

Table 4 illustrates the significant differences in parameters found in the 6 target species.

Avian enteric parasites

We examined 243 fecal samples (coffee=130; forest= 113) from 39 species (coffee=27; forest=27) of birds. The parasites examined were divided into 5 categories: coccidia, cestode, trematode, acanthocephalan and nematode. Infection (samples positive for any category of parasite/all samples examined) was 51% and 46% for coffee and forest respectively. Table 5a illustrates the percent infection by parasite category by habitat type for all species examined. To highlight the trends found, Table 5b illustrates

the percent infection specifically for four species which were abundant in both habitat types. Only one statistical significant result was found by habitat type, in which CCR had a higher level of infection with coccidian parasites in forest (67%) than coffee (21%). Other associations included: Season and infection with trematodes (Dry 20% > Wet 0%; p=0.0182) and Season and infection with nematodes (Dry 60% > Wet 11%; p=0.0101) for OBNT. For all other species there was not enough data or the associations were not significant. There were no significant differences in the diversity of parasites score between habitat type, season or reproductive season for all species pooled. Larvated eggs of *Syngamus trachea*, a nematode typically associated with chickens, were found in three CCR's, and one OBNT captured in a coffee plantation and a WTR captured in forest.

Avian ectoparasites

The mites collected from the wing feathers (and utilized for the ectoparasites score) were identified as either *Pterolichus obtusus*, (Fa: Pterolichidae), *Trouessartia* sp. (Fa: Trouessartiidae), *Pterodectes n. sp*. (Fa: Proctophyllodidae *Falculifer sp. nr. dinoceras* (Fa: Falculiferidae). Lice collected were identified as either *Sturnidoecus caligineus* (Fa: Philopteridae; Carriker, 1903), *Brueelia sp.* (Fa: Philopteridae), or *Myrsidea sp.* (Fa: Philopteridae). Two burrowing mites were identified as *Eutrombicula batatas* and *Blankaartia sinnamaryi* (Fa: Trombiculidae; Floch & Fauran, 1956) the latter of which was always associated with firm, round, fibrotic inflammatory skin lesions. Ticks were identified as *Ixodes spinipalpis* or *Amblyoma* sp. (Fa: Ixodidae) larvae which could not be identified to species (Hadwen & Nuttall, 1916). Interestingly, *Pterolichus obtusus* (Fa: Pterolichidae), a common mite of chickens, were collected from RCW and

HWRN. Parasitic flies were common on larger birds (>40 g) and were identified as Fa: Hippoboscidae.

Avian Hemoparasites

We examined 1,124 blood smears from 71 species of birds. Only two hemoparasites were detected, *Haemoproteus* sp. and microfilaria.. Of all the birds examined, 16% (n=179) of 23 species were infected with one of the two parasites. There were no significant associations between the various factors tested and presence of microfilaria. Table 6a illustrates the prevalence and species in which hemoparasites were found, and 6b illustrates only the birds in which *Haemoproteus* was found, by families. Since a difference by habitat type was found for WEGS, we then performed Chi-square analyses to determine if there was a difference by specific sampling site. The proportion of negatives and positives did differ by sites (p=0.0150), such that Zapote = Vargas (100%) > Joel (92%) > Giberth (86%) > Alvaro (75%) > Nenes (71%) > Pena (47%). We then reanalyzed the Chi-square without the Pena site which seemed the most different. The other sites were no longer significantly different (p=0.1210), and thus it appears that the Pena site is the one that is different from the others with the lowest level of infection.

Avian Serology

We tested 568 birds of 30 species (coffee=263; forest=242) for paramyxovirus via hemagglutination inhibition. Five birds (BCMotMot (2), RWWRN, WEGS and BTSL) were positive (<1%). Four of those birds were captured on coffee plantations and one (WEGS) was captured in forest. Whenever we had plasma remaining from paramyxovirus testing, we tested for *Mycoplasma gallisepticum* and *M. synoviae* (24

species, 265 individual birds; coffee=101; forest=141). Tables X and Xb detail the avian community tested for paramyxovirus and *Mycoplasma spp.*

*Mycoplasma spp.*nucleic acid detection and Paramyxovirus amplification and isolation

Two hundred and twenty choanal and cloacal swabs were collected during three sampling bouts from the six aforementioned target species and processed for DNA detection of *M. gallisepticum* and *M. synoviae* and virus isolation for Paramyxovirus. All samples were negative. Table 3 displays the distribution of samples obtained.

DISCUSSION

Avian Health Parameters

Body mass measurements (Wt/TarsusL and/or Wt/Wing) differed by reproductive season in RWWRN and RCW. Differences in Wt/Tarsus length body mass index were noted in RWWRN and RCW (higher in Non-Breeding). Body weight alone cannot be used as a measure of health, as it incorporates variation in body size (due to gender, age, etc) and condition (e.g. fat reserves) and thus weight is typically regressed on a size measure (19). Additionally, a significant difference in body condition score were noted in LTM (Non-Repro>Repro seasons; Dry>Wet), WEGS (Dry>Wet) and RWWRN (Dry>Wet). Thus, whenever a statistically significant difference was noted for any body mass or body condition score, the trend was for a bird to be in better condition in the Non-Repro and Dry seasons. We chose to use pectoral muscle mass, as opposed to fat reserves, as a measure of body condition because fat reserve scores are primarily utilized in migrants or in birds that live in extreme climates and do not apply themselves well to

tropical species (19). While it is easy to explain why body condition might be scored higher during the Non-Breeding season, as nesting and reproductive activity can place a high energy demand and negatively affect body condition, it is more difficult to explain seasonal differences, although clearly seasonal differences in food abundance exist. The three species for which significant differences were noted (LTM, RWWRN and WEGS) represent three feeding guilds (frugivory, insectivory and omnivory respectively) and thus, any explanation related to food availability would have to relate to all three food resources or involve some shift in resource acquisition (51). Indeed, the dry season in San Luis coincides with fruiting of trees primarily utilized by LTM's and there is some evidence that even insectivorous birds partake in the fruits available during December-March, when strong winds decrease the activity of arthropods (51). There was a significant difference in body weight for LTM (F>M) as has been previously reported (14). Also as expected, age was a significant factor for body weight of WEGS (Adults>Juv).

The feather mites identified in this study are not thought to have a detrimental effect on the health of birds, although some controversy exists regarding the damage that feather mites can cause to the feather pith or while gnawing through the calamus, weaking the feather prematurely (40). Indeed, I observed, for example, juvenile CCRs and WEGS with high feather mite loads and poor feather condition, much like chickens maintained in high-density corrals. Significant factors that affect loads of arthropods in hosts include intrinsic features such as species susceptibility, age, gender and immune system status. In addition, extrinsic factors, such as salt spray, have been investigated (12). Ectoparasite scores varied significantly in RWWRN (Wet>Dry; Non-

Repro>Repro), WEGS (Forest>Coffee), OBNT (Forest>Coffee) and LTM (Juv>Adult). The latter is not surprising, given that juveniles, having recently been more exposed to conspecifics might have higher loads of ectoparasites, as all of the feather mites identified in this study spend their entire lives on the host and are transmitted laterally (8). Additionally, solitary birds have little contact with conspecifics during the non-reproduction period. Another explanation suggested is that juvenile feathers are more heavily coated in oil and dandruff, which are the primary sources of food for non-parasitic mites (12, 40). Probably the single most important factor influencing ectoparasites loads is the molting pattern of birds. Molting allows for complete clearance of feather mites, and thus, the number of mites should be highest immediately before the molting period. Examining our records for molting patterns and reported information on molting patterns of birds in Monteverde, one would expect the highest accumulation of arthropods in May-August, as the peak of molting occurs in August-September, which coincides with the differences we noted (51). As opposed to birds in temperate zones, the molting pattern of Neotropical birds appears to overlap significantly with reproduction, and some have suggested that this overlap is due to a protracted molt period, to "spare" the high energy cost of molting, to allow for a higher level of immune responsiveness necessary in regions with higher load of parasites and pathogens (15, 30, 34). Habitat differences for feather mite load in WEGS and OBNT (both Forest>Coffee) perhaps could be explained by the microclimatic preferences of the mites themselves. The feather mites observed in these birds prefer cooler, more humid environments and reports that demonstrated the migration of feather mites from the dorsal to the ventral aspect of the wings of sun-bathing cormorants further supports this theory (13, 40). The chewing lice

recovered from wild birds were consistent with previous records of chewing lice on Neotropical birds (26). Chewing lice can cause significant irritation and in high numbers, can adversely affect the health of individual birds (26). *Pterolichus obtusus*, a feather mite of chickens was found in two species of birds (RCW and RWWRN) captured in coffee plantations. If present in high numbers, this mite can cause irritation and foraging disruption (9). Chickens were never processed during the same time periods as wild birds, and thus contamination by researchers is unlikely. However, these feather mites have been found in high numbers in chickens in the region and wild birds have been observed feeding and roosting in areas where chickens frequent. *Pterolichus obtusus* can live off its primary host for several days and thus, it is possible that sharing of dust baths, or other areas of high-density by wild birds and chickens could lead to temporary infestation of wild birds with chicken-associated mites (40)(Barry O'Connor pers. comm.).

Avian Enteric Parasites

There is currently much interest in understanding how non-lethal effects of pathogens and parasites affect animal populations and communities. Patterns of parasitism in bird populations, much like all wildlife are influenced by host density, behavior, intraspecific and interspecific contact rates, diet, and home ranges, all of which can be affected by human-associated changes to the landscape (17). Because assessing severity of infection from fecal egg and larvae counts is inappropriate, we are not making any inferences about the effects of parasites on the health of individual birds. We were most interested in the percent of infected birds, particularly in those that occurred in both habitats and the "parasite diversity score" which might illustrate whether birds inhabiting

human-disturbed areas harbored a larger diversity of parasites. Whereas, superficially, the above trend was noted, our results were not statistically significant. The only bird that harbored more coccidian parasites in one habitat than another (CCR: Forest > Coffee) is a highly mobile species, often foraging in open areas and breeding in forest fragments, and thus could not be considered to represent any one of the sites we sampled (47). The shedding of coccidian oocysts by Passerines follows a clear circadian rhythm, and differences in infection have been attributed to this cycle; however, since we captured all birds in the morning, we do not believe this factor affected our results (27). The other statistically significant results are easily explained by the seasonality associated with the life history of nematodes and trematodes. Most trematodes have complex life cycles requiring two intermediate hosts in which the parasites develop before they become infective for the final bird host and thus their seasonal distribution may be attributed to their intermediate host. Regarding nematodes, the seasonality found in this study may seem counterintuitive to the typical pattern of trichostrongyle nematodes, as they are typically found in higher numbers during "wet" periods, as the eggs do not develop in the environment under dry conditions. However, the leaf litter in Monteverde is rarely dry and it is possible that the wet season might be too wet and cause egg and larvae mortality. Further studies that examined the infection rates of OBNT's specifically during different seasons would be needed. Because trichostrongyle nematodes can cause significant chick mortality and decrease available energy for egg laying by adult birds (16), it would be particularly interesting to determine if the prevalence of these parasites during the wet season affects their fitness in the next breeding season.

Avian Hemoparasites

Avian hemoparasites have been a recent focus of disease ecology, as some have hypothesized that they play a role in sexual selection (28). However, information regarding the distribution and prevalence of these parasites as well as their effect on individuals as well as populations is still patchy (21). Our results were consistent with previous reports of the presence and prevalence of *Haemoproteus* spp. in passeriform birds, although we apparently report new records for some species (3, 4). Regarding the higher prevalence of infection with *Haemoproteus* during the Non-Breeding season for OBNT, Ricklefs found an association between lower parasite prevalence and incubation time. He suggested that species with long incubation periods generally had a lower prevalence of parasites because their immune systems were somehow better "prepared" and thus more resistant to infection (41). However, these theories would need to be specifically examined, as it is likely that information available on prevalence is heavily skewed by capture period.

In 1992, Young reported an 11% prevalence of blood parasite infections in birds in 10-20 yr old secondary and primary forest of the Monteverde region, from 479 birds of 60 species captured during a 1 year period. They reported two cases of *Plasmodium* spp. (WEGS and YFGQ), two cases of *Leucocytozoon* spp. (WTHR and PHV) and 1 case of *Trypanosoma* spp. (WILW). We were surprised that they obtained such a high diversity of hemoparasites, particularly as they sampled at a higher altitude (although it is not specifically clear from their report where in Monteverde they sampled), where presumably the density and diversity of vectors is lower (50). In particular, we question their *Plasmodium* spp. identification, as we obtained several samples with immature

stages of *Haemoproteus* spp., which could easily be confused with *Plasmodium spp*. Our results for OBNT further contradict Young's findings, as they report a higher prevalence of infection during the wet season, which corresponds to the Breeding season of most of the birds they examined. It would be interesting to review whether the anecdotal weather differences cited by Monteverde researchers are substantiated by data and could have affected vector biology enough to create these differences.

A significant habitat type difference in the prevalence of infection with *Haemoproteus* was detected for one species (WEGS); however, future research that includes details on the specific species of *Haemoproteus* infecting these birds and the abundance of the vector (*Culicoides sp*. or hippoboscid flies) would be needed to best understand the mechanism behind this difference. Both vectors are present and were observed during our study. Anecdotally, *Culicoides spp*. was encountered with more frequency in coffee plantations than in forest. It is also important to mention the limitations of describing prevalence and intensity of infection based on examinations of blood smears. *Haemoproteus*, for example, undergoes asexual reproduction (schizogony) in non-circulating cells, such as hepatocytes (liver cells), and the only stages of development found in circulating blood cells are gametocytes (the male, and female infective stages) (1). Thus, a host may be infected, but not have any visible parasites in circulating blood cells. Additionally, our method of capture (mist netting) assumes a bird is healthy enough to fly, thus birds undergoing the "crisis phase" of *Haemoproteus* infection (corresponding to peak parasitemia and peak physiologic stress) might have been excluded, underestimating true prevalence. Lastly, the examination of blood smears is a tiresome process that can lead to significant operator error.

Although Bennett et al found no association between body mass and prevalence of infection with hemosporidian parasites of Passerines, it is likely that *Haemoproteus* has a significant effect on the fitness of an individual, and possibly on a population, although this has not been specifically studied in most wild bird species, including WEGS (2)

Infections with *Haemoproteus* tend to be chronic, with relapses of parasitemia associated with periods of stress or food shortage (1). Anecdotally, it is interesting to note that of the birds in our study that died as a result of blood collection (n=4), three were WEGS (the species with the highest *Haemoproteus* prevalence, 76%) infected with *Haemoproteus*, leading one to theorize that the stressors of handling and blood collection on an already-anemic and compromised bird would predispose it for mortality. Unfortunately, we did not measure the hematocrit (percent red cell) of the birds in this study.

Serology, Virus isolation and DNA detection

We detected paramyxovirus antibodies in five birds and were unable to isolate virus from any of the cloacal swabs we collected. Thus, we are unable to make any inference about the differences in paramyxovirus exposure and habitat type. Similarly, we did not detect antibodies against *Mycoplasma spp.* or detected *Mycoplasma* DNA. We can rule out sample handling and laboratory methodology because we always utilized chickens from the region as "controls", for which we both detected antibodies and DNA.

The prevalence of paramyxovirus in wild Passerines is very low (35, 44, 46). As part of an investigation following an NDV outbreak in commercial chickens, Goodman

and Hanson sampled > 800 birds from various regions in Costa Rica and isolated parmyxovirus-2 from a single finch, a wren and a chicken from three different localities (18). Based on our results, it appears that this population of wild birds has a very low exposure to avian paramyxovirus.

Although the data is scarce, it appears that avian paramyxovirus-2 (APMV-2) might be the type most prevalent in Passeriformes and thus further studies that include larger sample sizes (to account for low prevalence) and testing for both types of AMPV are recommended (25). In addition, although the hemagglutination inhibition (HI) test is still the most widely used method due to its simplicity and ease of interpretation, its sensitivity is inferior to blocking Enzyme-linked Immunosorbent Assay (ELISA) (43). We did address the limitations of testing for antibodies by surveying our target species for antigen (either paramyxovirus through virus isolation or Mycoplasma through PCR). We were unable to obtain any evidence of antigen, although, once again, our capture method and the age structure of our sampled individuals might play a role in the results.

Similarly, we failed to detect birds that had antibodies to either *Mycoplasma gallisepticum* or *M. synoviae*. Other than the recent emergence of *M. gallisepticum*, and *M. sturni* in housefinches (*Carpodacus mexicanus*), and European starlings (*Sturnus vulgaris*) and other North American Passerines respectively, the prevalence of Mycoplasma infections, particularly those that might cause disease in Passerines is relatively unknown (16, 29). All three of these diseases require direct contact for transmission. Given the relatively high prevalence of paramyxovirus *and Mycoplasma spp*. in the chickens we surveyed that live in the shade-grown coffee plantations where

we were capturing birds, if direct contact between chickens and wild birds was common, we expected to see some evidence in susceptible wild birds. Given the low prevalence of these diseases in Passerines, further studies in which larger sample sizes and more sensitive methodology is utilized might be needed to elucidate habitat type differences.

CONCLUSIONS AND FUTURE DIRECTIONS

As aforementioned, shade-grown coffee plantations appear to have a variety of benefits for avian communities. On the other hand, shade coffee plantations are often surrounded by intensive agricultural land, and are thus "islands" of food and available habitat amidst a sea of unsuitable environments. The decrease in available wetlands has created concern amongst waterfowl managers that concentrations birds in higher densities could affect the transmission and severity of common waterfowl diseases such as avian cholera or botulism(6). This should be especially true during periods of migration when the rate of arriving susceptible individuals is high. Similarly, shade coffee plantations, which potentially pose other characteristics which tend to promote the introduction or persistence of diseases (impoverished biological diversity, comprised of large areas susceptible to "edge effect", colonized by exotic species, and harboring high rates of generalist species) are likely to concentrate wild birds in small areas. Thus, we hypothesized that birds inhabiting shade-grown coffee plantations would have lower health indicators and higher prevalence of pathogen and diversity when compared to those inhabiting forest fragments.

Generally, we have disproved our hypothesis. In the case of some pathogens, temporal scale, sample size, or testing logistics have limited our ability to explore our

theory as completely as possible. However, in cases where this was not a limitation, it appears that our suggested mechanisms (the introduction of chickens, the artificial concentration of wild birds, etc) have not played a role in changing the disease dynamics sufficiently to increase the prevalence and diversity of pathogens in avian communities living in shade-grown coffee. As mentioned before, at least two groups of pathogens deserve further attention: *Hemoproteus* and the diversity and identify of some parasites.

One alternative explanation is related to trade-offs—these plantations offer birds superb, constant food subsidies in the form of, for example, crops, planted fruiting trees and a variety of arthropods associated with crops and it has been suggested that shade coffee might act as refuges in times of low food availability in nearby fragments (20). In addition, preliminary studies indicate that foraging behavior of birds in shade coffee changes significantly. One of the landmark features of Neotropical birds in Monteverde is their participation in mixed species flocks, in which a variety of unrelated species feed and travels together (51). This behavior is logically thought to be related to predator avoidance. However, there is much less flocking behavior in birds in shade-grown coffee plantations. I have personally observed species which are known to participate in flocking behavior in forested habitat, feeding solitarily on a regular basis and at least one study has come to the same conclusion (39). Logically, coffee plantations are more "open" than forest and perhaps this allows single birds to monitor for predators more easily without the aid of other birds. Additionally, birds seem to be more homogenously distributed throughout coffee parcels, when compared to forest fragments, and perhaps the entire parcel can be viewed as "one large feeding flock". If that is the case, two significant stressors (food availability and concern over predators) might be lessened for

birds that inhabit coffee plantations, allowing energetic resources to be shifted towards immunity. Measuring stress and its effect on an individual or a population is the Holy Grail for most wildlife disease investigators. The level of coarseness of our health parameters may not be able to detect these subtle differences. However, procedures which could quantify avian immunity in small birds, with relatively non-invasive methods are in development and may be the next wave in determining fine habitat type differences (31). Finally, Gillespie summarized my thoughts as:

"*Our understanding of how anthropogenic habitat change alters wildlife disease dynamics is in its infancy. Our comprehension of this interplay will be greatly improved by future research that investigates how anthropogenic habitat disturbance affects the rates and patterns of parasite transmission within and between species, and how such changes affect the performance of host populations. Identifying risk factors for disease transmission will improve the ability of conservationists to make rational decisions about the risks and benefits of……..…….."* sustainable agricultural landscapes and other management activities (17).

Table 1. Eight sampling bouts were realized from 2005-2008 representing a variety of seasonal and reproductive seasons.

Sample Bout	Dates	Sites	Season	Reproduction
1	July-August 2005	Z, P, G	Wet	Breeding
2	Nov-Dec, 2005	Z, N, P	Dry	Non-Breeding
3	March-April, 2005	G, N, Z, P, A, V	Dry	Breeding
4	May-July, 2006	G, A, Z, P, N, J, V	Wet	Breeding
5	Feb-March, 2007	G, A, Z, P, N, J	Dry	Breeding
6	June-July, 2007	G, A, Z, P, N, J	Wet	Breeding
7	Sept-early Nov, 2007	G, A, Z, P, N, J	Wet	Non-Breeding
8	mid Nov-2007-Jan-2008	G, A, Z, P, N, J	Dry	Non-Breeding

Table 2. Six focal species were chosen for additional analyses. Their general characteristics are summarized herein.

Common name and abbreviation	Scientific name	Foraging guild	Foraging location	Average Body weight	Breeding months
Rufus and white wren (RWWRN)	*Thryothorus rufalbus* (Fa: Troglodytidae)	Insectivore	Ground	25.5 g	April-August
Rufous capped warbler (RCW)	*Basileuterus rufifrons* (Fa: Parulidae)	Omnivore	Undergrowth-Middle canopy	12.3 g	April-July
Blue-crowned Motmot (BCMotMot)	*Momotus momota* (Fa: Momotidae)	Omnivore	Middle canopy	114.0 g	March-May
Long-tailed Manakin (LTM)	*Chiroxiphia linearis* (Fa: Pipridae)	Frugivore	Middle canopy	19.7 g	February-May
Orange-billed Nightingale-Thrush (OBNT)	*Catharus aurantiirostris* (Fa: Turdidae)	Omnivore	Undergrowth-Middle canopy	29.3 g	March-August
White-eared Ground-Sparrow	*Melozone leucotis* (Fa: Emberizidae)	Omnivore	Ground-Undergrowth	43.1 g	April-June

Table 3. Distribution of choanal and cloacal samples obtained for *M. gallisepticum* and *M. synoviae* DNA extraction and paramyxovirus isolation. All samples were negative.

BIRD SPECIES	**TOTAL**	**COFFEE**	**FOREST**
BCMOTMOT	17	13	4
LTM	59	14	45
OBNT	40	21	19
RWWRN	21	8	13
WEGS	43	30	13
RCW	27	8	22
TOTALS	**220**	**94**	**116**

Table 4. Parameters which indirectly reflect the health of wild birds were measured and analyzed for differences based on habitat type, season, reproductive season and habitat*season. Significant differences are indicated herein. Whenever a difference was noted, the direction of the difference and adjusted least square means is represented. We were able to determine age and/or gender in two of the six target species. For those, the statistical analysis was repeated, considering age and gender.

Species	**Wt/Tarsus L**	**Wt/Wing Cord**	**Body Condition**	**Ectoparasite Score**
LTM	M>F (p=0.0201)	NS	Dry>wet (p=0.0097) NonRepro>Repro (p=0.0003)	Juv>Adult (p=0.0075)
RWWRN	NS	NonRepro>Repro (p=0.038)	Dry>wet (p=0.0010)	Wet>dry (p=0.014) NonRepro>Repro (p=0.0049)
RCW	NonRepro>Repro (p=0.0042)	NonRepro>Repro (p=0.0055)	NS	NS
WEGS	NS	NS	Dry>wet (p=0.025)	Forest> Coffee (p=0.031)
OBNT	NS	NS	NS	Forest>Coffee (p=0.0095)

Table 5. Percent of endoparasite infection, as determined from examining 243 fecal samples from 39 species, by parasite category in coffee and forest habitats.

Parasite	**Percent of Infection**	
	Coffee (n=130)	**Forest (n=113)**
Coccidia	31%	39%
Cestode	12%	3%
Acanthocephalan	4%	2%
Trematode	4%	2%
Nematode	17.69	8.85

Table 5b. Percent of endoparasite infection of four species which occurred in high abundance in both habitat types examined (coffee vs. forest), by parasite category in coffee and forest habitats; C=coffee; F=forest. The p value for the only statistically significant association is provided.

Parasite	**Percent Infection**							
	CCR (C) (n=19)	**CCR (F) (n=6)**	**OBNT (C) (n=17)**	**OBNT (F) (n=15)**	**RWWRN (C) (n=14)**	**RWWRN(F) (n=8)**	**WEGS (C) (n=11)**	**WEGS (F) (n=9)**
Coccidia	21%	67% (p=0.0368)	35%	20%	79%	100%	9%	44%
Cestode	26%	0%	24%	7%d	14%	0%	0%	0%
Acanthocephalan	5%	0%	0%	0%	0%	0%	18%	11%
Trematode	0%	0%	6%	0%	0%	0%	0%	11%
Nematode	53%	33%	12%	27%	14%	0%	0%	0%

Table 6a. The total number of samples examined per species and the proportion of individuals infected with hemoparasites is illustrated herein.

Bird Species	*Haemoproteus* sp.	Microfilaria
AFLY (n=1)	0	0
BASH (n=1)	0	0
BBFL (n=1)	0	0
BCMotmot (n=34)	0	0
BGTAN (n=6)	17%	0
BHNT (n=1)	0	0
BORI (n=4)	75%	0
BRJA (n=1)	0	0
BTSL (n=12)	17%	8%
BWOC (n=5)	0	0
BWW (n=2)	0	0
CANW (n=3)	0	0
CBT (n=3)	67%	0
CCR (n=102)	17%	2%
CQDV (n=1)	100%	0
DCFL (n=20)	0	0
ERF (n=1)	0	0
ETUC (n=11)	0	0
GCW (n=11)	36%	0
GKIS (n=2)	0	0
GSAL (n=2)	0	0
HWRN (n=15)	7%	0
LELA (n=1)	0	0
KBTU (n=1)	0	0
KTYW (n=4)	0	0
LGNT (n=4)	75%	0
LTM (n=161)	0	0
OBNT (n=114)	10%	0
MELA (n=1)	0	0
OSFL (n=4)	0	0
OVN (n=8)	0	0
OWOC (n=7)	0	0
PHV (n=1)	0	0
PWRN (n=23)	9%	0
MTR (n=2)	0	0
MTYR (n=1)	0	0
OBFL (n=23)	0	0
RBWR (n=1)	0	0
RBPS (n=2)	50%	0

RCAT (n=12)	0	0
RCW (n=69)	4%	1%
REVI (n=2)	100%	0
RWOC (n=40)	0	0
RWWRN (n=59)	0	0
SRTAN (n=1)	0	0
SWOC (n=4)	0	0
STHR (n=22)	9%	0
STTA (n=1)	0	0
SWOC (n=2)	0	0
TENW (n=1)	0	0
WEGS (n=123)	76%	0
WEW (n=1)	0	0
WILW (n=7)	0	0
WTDV (n=12)	0	0
WTHR (n=13)	8%	0
WTR (n=32)	13%	0
WWOC (n=1)	100%	0
WWPWEE (n=2)	0	0
YBC (n=3)	33%	0
YBEL (n=3)	0	0
YBFL (n=3)	0	0
YFGQ (n=10)	0	0
YFL (n=1)	0	0
YMFL (n=1)	0	0
YGV (n=13)	85%	0
YTBF (n=16)	56%	0
YTEU (n=64)	0	0
YCEU (n=3)	0	0
YTV (n=2)	0	0
YTYR (n=3)	0	0

*Abbreviations are as follows: Alder Flycatcher=AFLY; Baltimore Oriole=BORI; Barred Antshrike=BASH; Barred Woodcreeper=BWOC; Black-and-white warbler=BWW; Black-headed Nightingale thrush=BHNT; Blue-crowned Motmot=BCMotmot; Blue-gray Tanager=BGTAN; Boat-billed flycatcher=BBFL; Brown Jay=BRJA; Buff-throated Saltator=BTSL; Canada Warbler=CANW; Chiriqui Quail-Dove=CQD; Clay colored robin=CCR; Common Bush-Tanager=CBT; Dusky-capped Flycatcher=DCFL; Emerald Toucanet=ETUC; Eye-ringed Flatbill=ERF; Golden-crowned warbler=GCW; Grayish Saltator=GSAL; Great Kiskadee=GKIS; House Wren=HWRN; Keel-billed Toucan=KBTU; Kentucky Warbler=KTYW; Lesser Greenlet=LGNT; Long-tailed Manakin=LTM; Mistletoe tyrannulet=MTYR; Mountain Elaenia=MELA; Mountain Robin=MTR; Ochre-bellied flycatcher=OBFL; Olivaceous Woodcreeper=OWOC; Olive-striped Flycatcher=OSFL; Orange-bellied trogon=OBTR; Orange-billed Nightingale-Thrush=OBNT; Ovenbird=OVN; Philadelphia Vireo=PHV; Plain Wren=PWRN; Red-crowned Ant-tanager=RCAT; Red-eyed Vireo=REVI; Roufus-breasted wren=RBWR; Ruddy Woodcreeper=RWOC; Rufous-browed Peppershrike=RBPS; Rufous-capped Warbler=RCW; Rufus-and-white Wren=RWWRN; Scarlet-rumped Tanager=SRTAN; Silver-throated tanager=STTA;

Streaked-headed Woodcreeper=SWOC; Swainson's Thrush=STHR; Tennessee Warbler=TENW; Wedge-billed Woodcreeper=WWOC; Western Wood-Pewee=WWPWEE; White-eared Ground-Sparrow=WEGS; White-throated Robin=WTR; White-tipped Dove=WTDV; Wilson's warbler=WILW; Wood Thrush=WTHR; Worm-eating Warbler=WEW; Yellow bellied flycatcher=YBFL; Yellow Tyrannulet=YTYR; Yellow-bellied Elaenia=YBEL; Yellow-billed Cacique=YBC; Yellow-crowned Euphonia=YCEU; Yellow-faced Grassquit=YFGQ; Yellow-green vireo=YGV; Yellowish Flycatcher=YFL; Yellow-Margined Flycatcher=YMFL; Yellow-throated Brush finch=YTBF; Yellow-throated Euphonia=YTEU; Yellow-throated Vireo=YTV.

Table 6b. Out of the 71 wild bird species examined, 23 species were found to be infected with *Haemoproteus* spp. Asterisk denotes Neotropical migrants.

Family	Scientific Name	Bird Code	Common Name
Thraupidae	*Thraupis episcopus*	BGTAN	Blue-gray Tanager
	Chlorospingus opthalmicus	CBT	Common Bush-Tanager
Icteridae	*Icterus g. galbula*	BORI	Baltimore Oriole*
	Amblycercus holosericeus	YBC	Yellow-billed Cacique
Emberizidae	*Saltator maximus*	BTSL	Buff-throated saltator
	Melozone leucotis	WEGS	White-eared Ground-Sparrow
	Atlapetes gutturalis	YTBF	Yellow-throated Brush finch
Turdidae	*Turdus grayi*	CCR	Clay-colored Robin
	Turdus assimilis	WTR	White-throated Robin
	Catharus ustulatus	STHR	Swainson's Thrush*
	Catharus aurantiirostris	OBNT	Orange-billed Nightingale-Thrush
	Hylocichla mustelina	WTHR	Wood Thrush*
Vireonidae	*Cyclarhis gujanensis*	RBPS	Rufous-browed Peppershrike
	Vireo flavoviridis	YGV	Yellow-green vireo
	Vireo olivaceus	REVI	Red-eyed Vireo*
	Hylophilus decurtatus	LGNT	Lesser Greenlet
Columbidae	*Geotrygon costaricensis*	CQDV	Chiriqui Quail-Dove
Parulidae	*Basileuterus culicivorus*	GCW	Golden-crowned Warbler
	Basileuterus rufifrons	RCW	Rufous-capped Warbler
Troglodytidae	*Troglodytes aedon*	HWRN	House Wren
	Thryothorus	PWRN	Plain Wren

	modestus		
	Thryothorus rufalbus	RWWRN	Rufus-and-white Wren
Dendrocolaptidae	*Glyphorhynchus spirurus*	WWOC	Wedge-billed Woodcreeper

Table 7a. Thirty bird species from San Luis, Costa Rica were tested for avian paramyxovirus-1 via HI. The name, and number of individuals tested, divided by habitat type is illustrated below.

Bird Species	**No. of individuals tested for paramyxovirus-1**
BBFL	1
BCMotmot	27
BGTAN	3
BORI	4
BRJA	1
BTSL	8
BWOC	1
CCR	94
DCFL	1
ERF	1
ETUC	9
GKIS	1
GSAL	1
LTM	5
MTR	3
OBNT	68
PWRN	2
RBPS	2
RCAT	10
RCW	2
RWOC	32
RWWRN	43
STHR	17
STTA	1
SWOC	5
WEGS	100
WTDV	11
WTHR	10

WTR	33
YTBF	11
YTEU	2

Table 7b. Twenty four species from San Luis, Costa Rica were tested for avian *Mycoplasma gallisepticum* and *M. synoviae* via HI. The name, and number of individuals tested, divided by habitat type is illustrated below.

Bird Species	**No. of individuals tested for Mycoplasma gallisepticum and M. synoviae**
BBFL	1
BCMotmot	15
BGTAN	2
BORI	1
BRJA	1
BTSL	3
BWOC	2
CCR	33
ETUC	4
GKIS	1
GSAL	1
LTM	2
OBNT	29
RCAT	4
RWOC	20
RWWRN	24
STHR	10
SWOC	2
WEGS	59
WTDV	7
WTHR	5
WTR	15
YTBF	1
YTEU	1

Appendix I. Common name, bird code and scientific name of bird species captured during a study of the diseases birds in two habitat types in San Luis, Costa Rica.

Alder Flycatcher	AFLY	Empidonax alnorum
Baltimore Oriole	BORI	Icterus g. galbula
Barred Antshrike	BASH	Thamnophilus doliatus
Barred Woodcreeper	BWOC	Dendrocolaptes certhia
Black-and-white warbler	BWW	Mniotilta varia
Black-headed Nightingale thrush	BHNT	Catharus mexicanus
Blue-crowned Motmot	BCMotmot	Momotus momota
Blue-gray Tanager	BGTAN	Thraupis episcopus
Boat-billed flycatcher	BBFL	Megarhyncus pitangua
Brown Jay	BRJA	Cyanocorax morio
Buff-throated Saltator	BTSL	Saltator maximus
Canada Warbler	CANW	Wilsonia canadensis
Chiriqui Quail-Dove	CQD	Geotrygon costaricensis
Clay colored robin	CCR	Turdus grayi
Common Bush-Tanager	CBT	Chlorospingus opthalmicus
Dusky-capped Flycatcher	DCFL	Myiarchus tuberculifer
Emerald Toucanet	ETUC	Aulacorhyncus prasinus
Eye-ringed Flatbill	ERF	Rhynchocyclus brevirostris
Golden-crowned warbler	GCW	Basileuterus culicivorus
Grayish Saltator	GSAL	Saltator coerulescens
Great Kiskadee	GKIS	Pitangus sulphuratus
House Wren	HWRN	Troglodytes aedon
Keel-billed Toucan	KBTU	Ramphastos swainsonii
Kentucky Warbler	KTYW	Oporornis formosus
Least Flycatcher	LFL	Empidonax minimus
Lesser Elaenia	LELA	Elaenia chiriquensis
Lesser Greenlet	LGNT	Hylophilus decurtatus
Long-tailed Manakin	LTM	Chiroxiphia linearis
Louisiana Waterthrush	LAWTHR	Seiurus motacilla
Mistletoe Tyrannulet	MTYR	Zimmerius vilissimus
Mountain Elaenia	MELA	Elaenia frantzii
Mountain Robin	MTR	Turdus plebejus
Ochre-bellied flycatcher	OBFL	Mionectes oleagineus
Olivaceous Woodcreeper	OWOC	Sittasomus griseicapillus
Olive-striped Flycatcher	OSFL	Mionectes olivaceus
Orange-bellied trogon	OBTR	Trogon aurantiiventris
Orange-billed Nightingale-Thrush	OBNT	Catharus aurantiirostris

Ovenbird	OVN	Seiurus aurocapillus
Philadelphia Vireo	PHV	Vireo philadelphicus
Plain Wren	PWRN	Thryothorus modestus
Plain Xenops	PXEN	Xenops minutus
Red-crowned Ant-tanager	RCAT	Habia rubica
Red-eyed Vireo	REVI	Vireo olivaceus
Roufus-breasted wren	RBWR	Thryothorus rutilus
Ruddy Woodcreeper	RWOC	Dendrocincla homochroa
Rufous-browed Peppershrike	RBPS	Cyclarhis gujanensis
Rufous-capped Warbler	RCW	Basileuterus rufifrons
Rufus-and-white Wren	RWWRN	Thryothorus rufalbus
Scarlet-rumped Tanager	SRTAN	Ramphocelus passerinii
Silver-throated tanager	STTA	Tangara icterocephala
Streaked-headed Woodcreeper	SWOC	Lepidocolaptes souleyetii
Swainson's Thrush	STHR	Catharus ustulatus
Tennessee Warbler	TENW	Vermivora peregrina
Unidentified Bird	UNBI	0
Wedge-billed Woodcreeper	WWOC	Glyphorhynchus spirurus
Western Wood-Pewee	WWPWEE	Contopus virens
White-eared Ground-Sparrow	WEGS	Melozone leucotis
White-throated Robin	WTR	Turdus assimilis
White-tipped Dove	WTDV	Leptotila verreauxi
Wilson's warbler	WILW	Wilsonia pusilla
Wood Thrush	WTHR	Hylocichla mustelina
Worm-eating Warbler	WEW	Helmitheros vermivorus
Yellow bellied flycatcher	YBFL	Empidonax flaviventris
Yellow Tyrannulet	YTYR	Capsiempis flaveola
Yellow-bellied Elaenia	YBEL	Elaenia flavogaster
Yellow-billed Cacique	YBC	Amblycercus holosericeus
Yellow-crowned Euphonia	YCEU	Euphonia luteicapilla
Yellow-faced Grassquit	YFGQ	Tiaris olivacea
Yellow-green vireo	YGV	Vireo flavoviridis
Yellowish Flycatcher	YFL	Empidonax flavescens
Yellow-Margined Flycatcher	YMFL	Tolmomyias assimilis
Yellow-throated Brush finch	YTBF	Atlapetes gutturalis
Yellow-throated Euphonia	YTEU	Euphonia hirundinacea
Yellow-throated Vireo	YTV	Vireo flavifrons

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CHAPTER 7

AVIAN COMMUNITY COMPOSITION OF SHADE COFFEE AND FOREST FRAGMENTS IN SAN LUIS, COSTA RICA

INTRODUCTION

Habitat loss and fragmentation in the Neotropics continue to be the leading threat to biodiversity (43). Like in most of the Neotropics, deforestation continues to be a problem in Costa Rica (16). The Costa Rican countryside, near Monteverde and outside the Monteverde Cloud Forest, Santa Elena and the Children's Eternal Rainforest reserves that give it its fame, is a series of forest fragment islands in an ocean of pasture for livestock. Although the private reserves support some of the most threatened, large-bodied (>50 g) avian endemics (e.g. Resplendent Quetzal, *Pharomachrus mocinno,* Three-wattled Bellbird, *Procnias tricarunculatus*), the protection they afford smaller-bodied, resident avian species is questionable. Janzen coined the term "gardenification" to emphasize a philosophical shift in conservation away from total reliance upon unpopulated preserves and towards the management of human-inhabited landscapes to improve biodiversity (13). Modern conservation approaches that follow the latter approach differ in that they emphasize the importance of 1) human-dominated landscapes, 2) ecosystem services, and 3) innovative finance mechanisms (39).

In particular since the 1980s, the field of sustainable agriculture has gained much momentum in Costa Rica to ensure that private land, while providing economic support for people, could be used to maintain habitat to support biodiversity and buffer protected areas. Coffee plantations managed under a floristically and structurally diverse canopy (henceforth, shade coffee) provides important habitat for biodiversity (26, 29, 30). Thus, shade coffee plantations are gardens tended by small farmers for economic benefit, while potentially playing a vital role in maintaining biodiversity. The benefits to biodiversity from shade-grown coffee systems are well documented and have been summarized in recent reviews (26, 31-34).

Most studies on shade coffee in Latin America have concentrated on diversity indices as a way to align these landscapes as surrogates for secondary forest (8, 9). Although useful, analyses that focus on diversity indices often ignore species composition. Additional approaches to describe communities utilize logistic regression to assess the relationship between environmental characteristics and species presence or absence. However, accurate inferences from these approaches require constant detectability across the environmental conditions of interest, but detectability of species often varies with respect to weather conditions, breeding phenology, and vegetation structure (27). MacKenzie *et al.* developed a likelihood-based approach for obtaining unbiased estimates of site-occupancy rates while accounting for capture when capture is imperfect (24). As MacKenzie points out, a potential consequence of ignoring capture is that the estimates of occupancy and other associated parameters can be biased, resulting in misleading inferences about a system, thus translating to inappropriate management decisions (23). Through this approach, model parameters are defined to represent

occupancy and capture probabilities, and covariates are then applied as predictors to explain variation in occupancy and capture (23). This approach was extended to model rates of local extinction and colonization, in addition to rates of initial patch occupancy and species detectability (henceforth, multi-season occupancy) (25). The multi-season occupancy model therefore may be used to evaluate differences in species turnover rates between shade coffee and unfarmed forest. Conservation organizations promote shade-grown coffee as forest surrogate habitat in Costa Rica and other Neotropical countries (15). However, uncertainty remains as to how biodiversity (e.g., avian community composition) in shade-grown coffee plantations compares with intact forest.

The objective of this study was to account for imperfect capture while comparing occupancy and turnover rates of avian communities from mist-net captures in shade-coffee plantations and unfarmed forest fragments in the region of Monteverde, specifically in the San Luis valley, Costa Rica. To our knowledge, this is the first study to apply the multi-season occupancy model to a multi-species system. In particular, we model capture (i.e., capture) probability based on netting effort, temporal variation, habitat type, weather patterns, and traits of species groups. We then model occupancy and change in occupancy based on habitat type, temporal variation, and traits of species groups. Finally, we provide recommendations for managers in the Neotropics for promoting avian community integrity in shade coffee plantations.

MATERIALS AND METHODS

Field Sites

This research took place in the San Luis valley, located in the Northwest region of Costa Rica approximately 7 km from the town of Santa Elena in the Monteverde region, in the province of Guanacaste. Seven field sites located near the town of San Luis (10 16'57,117" N 84 47'53,747" W) were chosen and are described herein. The sites were chosen to represent two habitat types (shade-grown coffee and forest), as part of a larger ecological study investigating habitat-associated diseases of wild birds. All sites are considered to be pre-montane wet forest (PMWF) (10). All sites were approximately 1.5 ha in size. Three were privately owned shade-grown coffee plantations and three were embedded within old secondary growth forest (>50 yrs; henceforth, forest).

Avian capture

Birds were captured with mist nets following standard methodology (4). Approximately 8-14 nylon mist nets (38 mm nylon mesh, 4 panels, 3 m high and either 6 or 9 m in length) were erected the evening before a capture day and maintained furled. The total net length was maintained equal across sites sampled. During each capture day, the nets were opened between 5-7 AM, and maintained open until 1-2 PM or until 10 birds not previously banded were captured per day. Table 1 details the capture schedule from 2005-2008. The nets were monitored every 30 min. Nets were opened on one of two alternating sites to avoid capturing in one site on two consecutive days, which may lead to a decrease in daily capture probability due to net shyness. Human activity was not permitted near the nets while they were open, other than to extract birds. The nets were closed early if it rained, or if it was >20 kmph as these conditions both affect capture rate

and endanger the welfare of individual birds. Birds were extracted from the nets and placed in disposable brown paper bags. Birds were processed immediately to minimize holding time. As birds were held for biological sample collection, they were processed within 20 min of extraction from the nets, and released near the point of capture. All birds were identified to species according to Stiles (42). To identify recaptures, we applied a unique combination of plastic color coded bands. All avian capture and handling techniques were reviewed and approved by the University of Georgia's Animal Care and Use Committee.

Community Composition Analysis

Modeling approach.-We used an information-theoretic approach to compare models of capture probability, occupancy, and change in occupancy between seasons. In particular, to compare models based on maximum likelihood estimation, we used Akaike's Information Criterion (AIC_c), which is a measure of parsimony that corrects for small sample size (1-3, 6). Models that are best at predicting the response (e.g., capture probability) with the fewest predictors will have the lowest AIC_c and are therefore the most parsimonious. We defined a confidence set of models as those with a $\Delta AIC_c \leq 4$, where ΔAIC_c equals the AIC_c of focal model minus that of the model with the lowest AIC_c value of the set (henceforth, top model). We applied the "Robust Design Occupancy" data type in Program MARK to generate AIC_c values and parameter estimates, including rates of capture, initial occupancy, extinction, and colonization (44). In cases where parameter estimates did not converge, we applied the Markov Chain Monte Carlo (MCMC) option in program MARK(7). After applying the MCMC option, we assessed convergence by the methods of Raftery and Lewis, using the default values

implemented in CODA (35, 37, 38). We compared such models using the Deviance Information Criterion (DIC), which is recommended over AIC for Bayesian approaches to parameter estimation from non-hierarchical models (41). As with AIC_c, we defined a confidence set of models as those with a $\Delta DIC \leq 4$

Here, we employ the multi-season occupancy approach, which is an extension of MacKenzie's single-season occupancy model (24, 25). The original model was a likelihood-based method for estimating site occupancy rates (i.e., fraction of landscape units where the focal species or group of species is present) when probability of detecting a species or group of species is less than 1. The model was extended to enable estimation of rates of extinction (i.e., probability that an occupied patch becomes unoccupied in the following season) and colonization (i.e., probability that an unoccupied patch becomes occupied in the following season). The model requires as input detection histories that include primary and secondary sampling occasions. We defined primary sampling occasions, among which, changes in site occupancy can occur, as individual seasons (see below for capture history details). Within each primary sampling occasion, we defined secondary sampling occasions, among which, site occupancy is assumed to remain constant, as individual sampling bouts (sampling bouts= the order in which each site was visited for a particular season). We believe that community-level shifts in site occupancy among sampling bouts were unlikely, and therefore this assumption was probably met.

Capture histories.-Based on preliminary analyses, captures were too sparse to enable the estimation of species-specific occupancy rates. We therefore combined capture information into species groups, or guilds, which were defined based on life history attributes. Given seven sites and 13 guilds, we developed a total of 91 site-guild capture

histories. Data were also too sparse to enable estimation of daily capture probabilities. We therefore combined captures across individual days for each sampling bout for each season, totaling 12 possible season-bout combinations (Table 1). The last three season-bout combinations were identical to the first, which allowed us to model change in occupancy between seasons 1 and 2, between seasons 2 and 3, and between seasons 3 and 1. We defined the dry, non-peak breeding season as the season for estimating initial occupancy, as this season had the most replication across sites, sampling bouts. Furthermore, the migratory guilds were assumed to be absent during season 3, the wet, peak-breeding season, which greatly limited the level of replication for that season.

Candidate model comparisons.-We first constructed a set of models for temporal variation in capture rates, which included all combinations of additive effects and an interaction between season and sampling bout. These models assumed constant rates of initial occupancy and change in occupancy between seasons. We compared this set of models with a null model that assumed capture rate was constant over time. Factors, if any, found in the top model from this set were included in all subsequent capture models.

We then constructed an additional set of capture models that incorporated the following a priori factors that we suspected could directly affect capture rates for understory forest birds in the Neotropics, in order of decreasing priority: 1) habitat (i.e. shade coffee or forest), 2) habitat-by-season interaction, 3) rainfall, 4) wind speed, 5) habitat-by-wind speed interaction, 6) foraging-place guild membership (i.e., low or high), 7) migratory guild membership, 8) net effort, and 9) habitat-by-net effort interaction. We only considered combined-factor models (e.g., rainfall and windspeed) if either 1) one of the factors was in the top 3 of the priority list, or 2) two or more factors were included in

the initial confidence set of models (henceforth, high ranking factors). This set of models included the top temporal capture model, which assumes that capture rates were constant across these other factors. The top model from this set was applied to all subsequent occupancy models.

Next, we constructed a set of initial occupancy models that incorporated the following a priori factors, in order of decreasing priority: 1) habitat, 2) diet guild membership, 3) foraging-place guild membership, and 4) migratory guild membership. Factors 2-4 included the respective habitat-by-guild interaction term. As with the capture models, we only considered combined-factor models that included high-ranking factors. The top model from this set was applied to all subsequent change-in-occupancy models.

We finally constructed a set of a priori change-in-occupancy models. Change in occupancy is comprised of two parameters: probability of extinction and probability of colonization between seasons. As we had no *a priori* reason to believe that these parameters would be affected differently by the factors of interest, we applied each factor of interest to both parameters when modeling change in occupancy. These models incorporated the following factors, in order of decreasing priority: 1) habitat, 2) season, 3) diet guild membership (i.e., frugivore, insectivore, or omnivore), and 4) forest-dependency guild membership, and 5) migratory guild membership. Factors 3-5 included the respective habitat-by-guild interaction. As with the previous model sets, we only considered combined-factor models that included high-ranking factors.

Model predictions.-We used the top model from each set for making predictions and inferences about patterns of avian guild occupancy. In particular, inferences about differences among levels within each factor were based on comparisons of 95%

confidence intervals or 95% Bayesian credibility limits (i.e., 2.5th and 97.5th percentiles from MCMC output) surrounding parameter estimates. We also used the "incidence function" introduced by Hanski 1994, which predicts the stationary occupancy rate at which site occupancy remains constant over time based on fixed rates of colonization and extinction (11). The multi-season occupancy model, in fact, is an extension of this incidence function (25). Finally, we estimated the number of seasons required to reach stationary occupancy.

RESULTS

Avian capture

Bird captures took placed from 2005-2008 during 8 sampling bouts, representing combinations of two biologically relevant factors: climatic cycle (i.e., alternating wet and dry seasons) and reproductive activity (peak breeding and non-peak breeding seasons) (Table 1). Including recaptures, we captured 1,561 individuals across 74 species, including 62 species in shade coffee and 47 species in forest (Table 2). We captured 27 species in shade coffee that were not captured in forest, 12 species in forest that were not captured in coffee, and 35 species in both habitats (Table 3).

Capture rate

A model with additive effects of season and sampling bout was the most parsimonious temporal capture model, and a season-specific model was also included in this confidence set (ΔAIC = 3). Capture rates were higher during the wet, peak breeding season when compared to the other two seasons (Graph 1). Although confidence

intervals overlapped, there was an increasing trend in capture probability across sampling bouts (Graph 1).

While assuming capture rate varied by season and sampling bout, a model with average wind speed was more parsimonious than models comprised of other factors or combinations of factors proposed to directly affect capture probability. This confidence set, however, contained 10 other models. In addition to wind speed, these models included the following factors and combinations of factors: interaction between habitat type and foraging-location guild, rainfall, habitat type, and net effort. Most importantly, this confidence set included a null model with only sampling bout and seasonal effect, which indicates uncertainty as to whether any of these direct factors are important for explaining variation in capture rates. Including the top model, however, wind speed was included in 6 of the models in the confidence set. Predicted capture rate increased, on average, from 50% to 70% as average wind speed increased from 0 to 11 kmph during the first sampling bout for the dry, non-peak breeding season (Graph 2).

Initial occupancy

While assuming that capture rate varied by season, sampling bout, and wind speed, a model with an interaction between habitat and the forest dependency guild dominated this confidence set of models (ΔAIC of next best model was 14). A model that assumed constant initial occupancy (i.e., null initial occupancy model) was also much less parsimonious than this top model (ΔAIC = 33). Occupancy estimates from this top model did not converge, but we confirmed the dominance of this model by obtaining the DIC values for this and the next-best model using the MCMC option. Initial occupancy rates (i.e., during the dry, non-peak breeding season) for the forest obligate

guild in shade coffee were lower than those for the forest facultative guild in either habitat type (Graph 3). Credibility intervals overlapped in comparisons of initial occupancy for the remaining habitat-guild combinations (Graph 3).

Change in occupancy

As with initial occupancy, a turnover model where extinction and colonization each varied by an interaction between habitat type and forest-dependency guild membership was the most parsimonious of the set. The only other model in the confidence set was one that assumed constant change in occupancy (i.e., null model for this set; $\Delta AIC = 1.6$). As both models exhibited convergence failures, we confirmed that the model with an interaction term was more parsimonious and in fact became the sole member of the confidence set according to their respective DIC values from the MCMC output ($\Delta DIC = 9$ for the null model of this set). Extinction rate between successive seasons (dry-non-peak-breeding, wet-non-peak-breeding, wet-peak-breeding) was greater for the forest obligate guild in coffee than for the forest facultative guild in forest (Graph 4). The 95% credibility intervals overlapped between the remaining pair-wise comparisons of habitat-guild combinations. Except for the forest obligate guild in coffee, colonization rates for the remaining habitat-guild combinations tended to be greater than extinction rates. Rates of change in occupancy (extinction rate plus colonization rate) tended to be greater in coffee than in forest (Graph 3). The mean stationary occupancy rate in forest is higher than that in coffee, but credibility intervals overlap extensively (Graph 3). Except for the forest obligate guild in forest, initial occupancy rates were within 10% of the mean stationary occupancy rate (Graph 3). Initial occupancy rates for all habitat-guild combinations were above the mean stationary occupancy rate, except the

forest obligate guild in forest (Graph 3). According to the model, the forest facultative guild reached stationary occupancy after 4 and 3 seasons, while the forest obligate guild reached stationary occupancy after 6 and 10 years in coffee and forest, respectively.

DISCUSSION

A recent study that examined avian assemblages in cacao and banana agroforestry systems concluded that these systems contained bird assemblages that were as abundant, species-rich and diverse as forests. The species composition of these assemblages was highly modified, however, in that there were fewer forest obligate species, more species associated with "open areas", and different dominant species, suggesting that management efforts should always include conserving forested habitat and highlighting the importance of examining specific community compositions (12). Indeed, in accordance with our prediction about seasonal changes in site occupancy by avian guilds in the Monteverde region of Costa Rica, our study indicates that although the apparent species richness was higher in coffee (n=61) when compared to forest (n=46), the community composition varied across habitats. Initial occupancy estimates indicated that forest obligates are more widely distributed across unfarmed forest fragments than across shade coffee plantations. This agrees with other studies that describe avian community composition across these habitat types in Costa Rica and other tropical countries (12, 20, 28, 40). The overall trends we found imply that forest obligate species colonize shade coffee at lower rates than forest fragments, and do not persist in coffee to the same extent as in forest fragments. Forest obligate species had lower interseasonal persistence in shade coffee plantations than in unfarmed forest fragments. Finally, extinction was

generally greater than colonization in both habitat types, and specifically, extinction rate of forest obligates was greater in coffee than extinction rate of forest facultatives in forest.

Previous studies have indicated that forest understory insectivores, understory bark insectivores and mid-story insectivores are rare or absent in coffee plantations (14). Indeed most of the forest obligates we captured fall under these categories (members of the Dendrocolaptidae: *Sittasomus griseicapillus, Glyphorhynchus spirurus*, and *Dendrocolaptes certhia*; antbirds in Thamnophilidae: *Thamnophilus doliatus*; members of the Tyrannidae: *Rhynchocyclus brevirostris*, and *Tolmomyias assimilis*). In general, the insectivorous guild is the one reported to be least represented (in terms of richness) in shade coffee plantations (14); however, our findings indicated that variation in site occupancy was better explained by level of forest dependency than by dietary associations. It is interesting to note, however, that of our list of forest obligates, 60% were also classified as strict insectivores. These findings are important for management and conservation. Shade coffee provides suitable habitat for forest facultative species, and the literature reviewing shade coffee demonstrates a positive effect on both biodiversity and abundance, but certain species will always be reliant upon forest fragments.

It has been proposed that the shorter stature of coffee habitat would allow for higher detectability, although most studies on abundance and richness in coffee plantations have not corrected for detectability (14). Our analysis indicated that capture rate did not differ between habitats. In fact, the habitat-specific capture models were as or less parsimonious than the temporal capture model that assumed capture rates were

equal between habitats. Thus, capture probability varied by season, such that it was highest during the wet, peak breeding period. This is likely due to the increase in activity during the breeding season to defend territories, build nests and take care of young. As most of the species we captured are sexually monomorphic, we could not analyze our data for relationships between gender-related activity (male territoriality or female nest activity) and season. Capture probability estimates also increased (50-70%) when average wind speed for the sampling bout increased (0-11 kmph). This is counterintuitive, as windy conditions lead to a decrease in bird activity (Hernandez-Divers, pers. obs.) and the motion of the nets in the wind should allow birds to see the net and thus decrease capture rate. A study examining the effect of wind speed on mist net captures reported an increase of 7-16% escape rate on high wind days when compared to low wind days, which would support our observations in the field (Hernandez-Divers pers. observations) (21). As wind speed was averaged over multiple days, the result may be an artifact if other unmeasured variables that more directly regulate capture rates were correlated with wind speed. We also found a general trend for capture probability to increase with each successive sampling bout, which may relate to increased efficiency and recognition of areas of high bird activity as the study progressed.

The higher abundance of birds in shade coffee plantations has been attributed to greater abundance of resident, rather than migrant guilds (45). Whereas we did not estimate actual abundance, we estimated individual capture rates by dividing the number of individual birds, by the total number of net hrs. While mean individual capture rate was 0.1 captures hr^{-1} higher in coffee than in forest, the confidence intervals overlapped (Table 2).

Environmental conditions vary dramatically in the Monteverde region with small changes in altitude, such that six life zones occur within a 600-m elevational range (10). Avian community composition often changes rapidly with elevation in the Neotropics, and specifically in Costa Rica, bird species composition changes substantially from 500-1000 m, with bird species richness declining dramatically above 1500 m (5, 47). In the Monteverde area, there is evidence of both high bird species turnover across elevations and declining bird species richness with elevation (46, 47). This has important implications for research. For one thing, extrapolations cannot be made within relatively short distances in Monteverde, due to significant differences of avian community composition. Thus, comparative studies dictate that field sites are located close to one another. Additionally, a large number of studies have focused on species found specifically in the Monteverde reserves (e.g. Resplendent Quetzals, Brown Jays, Black-faced Solitaires, Collared Red-starts, etc) and on features which characterize the avifauna in the Monteverde region (e.g. mixed species flock, foraging and social behavior, altitudinal migration, etc) (17-19, 36) . Yet, relatively little data has been collected on the avifauna just outside the protected zones. Lastly, much of the research has focused on single-species and has ignored important community questions, which, in turn, is tied to important conservation themes.

Komar (2006) reminds us that, in the end, the findings of studies of diversity and abundance in shade coffee plantations probably adhere to the principles of island biogeography (14). That is, larger patches of habitat are more likely to harbor more species than smaller patches (here patches could be shade coffee plantations), and similarly, patches closest to intact forest will receive richer and more abundant

colonization than patches further away (22). Thus, mixed conservation strategies that utilize shade coffee not as an absolute forest surrogate habitat, but rather as complimentary to connect and buffer remaining forest fragments are recommended.

Table 1. Birds were captured in seven sites (four shade-coffee plantations and three forest fragments) near San Luis, Costa Rica during 2005-2008. The biological season, sampling bout and sites sampled are illustrated herein.

		Dry											
		Non-peak breeding				**Peak breeding**				**Wet, peak breeding**			
Site		2004-5	2005-6	2006-7	2007-8	2005	2006	2007	2008	2005	2006	2007	2008
Shade coffee													
	Gilbert			1st			1st			1st	2nd	3rd	
	Joel			1st	2nd						1st	2nd	
	Alvaro			1st	2nd		1st				1st	2nd	
	Vargas						1st				1st		
Forest													
	Zapote	1st			2nd		1st			1st	2nd	3rd	
	Nenes	1st		2nd			1st				1st	2nd	
	Pena	1st			2nd		1st	2nd		1st	2nd	3rd	
	TOTAL	3		4	4		6	1		3	7	6	

Table 2. Species and number of individuals per species captured in shade coffee and forest sites near San Luis, Costa Rica during 2005-2008.

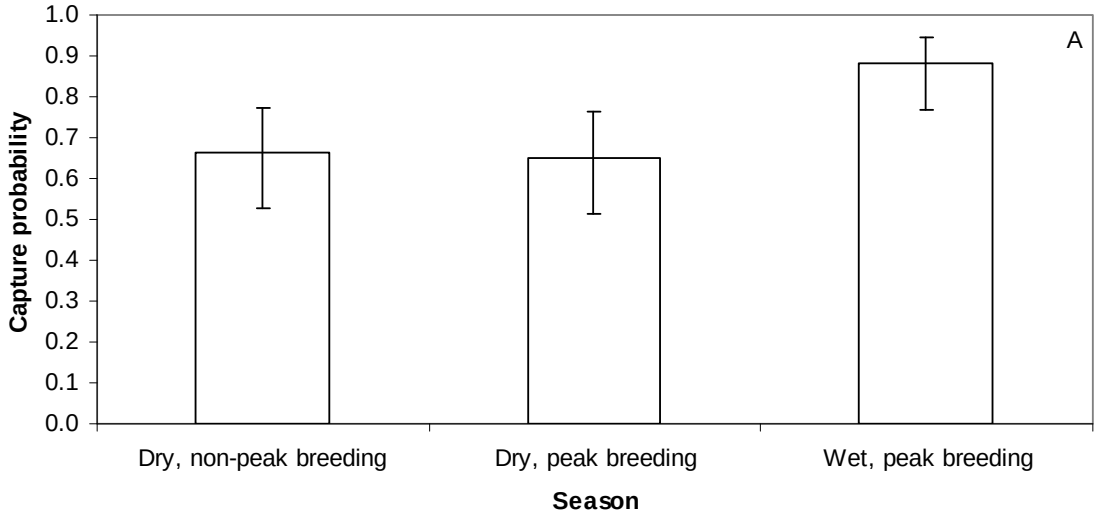
Coffee Sites			Forest Sites	
Name of Species	**Number of individuals Captured**		**Name of Species**	**Number of individuals Captured**
CCR	84		LTM	151
WEGS	79		OBNT	90
OBNT	73		WEGS	80
YTEU	66		RWWRN	62
LTM	49		RCW	55
RWWRN	49		RWOC	48
RCW	41		OBFL	33
PWRN	29		WTR	27
BCMotmot	26		GCW	25
YFGQ	26		CCR	23
STHR	24		RCAT	19
DCFL	22		BCMotmot	16
HWRN	19		STHR	13
YGV	13		YTEU	12
YTBF	12		WTHR	11
BTSL	11		CBT	10
OBFL	11		KTYW	10
WTR	11		OVN	10
WTHR	10		OWOC	10
ETUC	9		PWRN	10
WTDV	8		LGNT	9
RWOC	6		YTBF	8
WILW	6		BWOC	6
BGTAN	5		ETUC	5
MTR	5		WTDV	5
OVN	5		DCFL	4
SWOC	5		HWRN	4
BORI	4		WILW	4
LGNT	4		OSFL	3
MTYR	4		SWOC	2
YBC	4		WEW	2
YBFL	4		YTYR	2
AFLY	3		BHNT	1
BWW	3		BTSL	1
RBPS	3		BWW	1
TENW	3		CANW	1
BASH	2		ERF	1
CANW	2		KBTU	1
GSAL	2		OBTR	1
OSFL	2		PXEN	1
PHV	2		RBWR	1

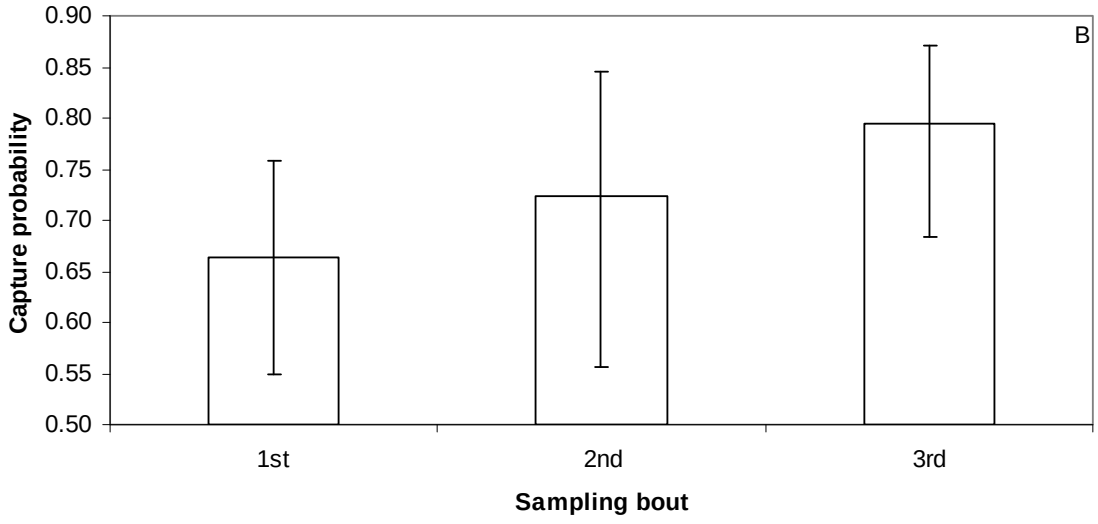
REVI	2		SRTAN	1
YBEL	2		TENW	1
YTV	2		YBC	1
BBFL	1		YFGQ	1
BRJA	1		YFL	1
CQD	1			
GCW	1			
GKIS	1			
KTYW	1			
LAWTHR	1			
LELA	1			
LFL	1			
MELA	1			
STTA	1			
WWOC	1			
WWPWEE	1			
YCEU	1			
YFL	1			
YMFL	1			
YTYR	1			
TOTAL	**769**		**TOTAL**	**778**
	1.33 birds captured per net hour			**1.2 birds captured per net hour**

Table 3. Species captured exclusively in shade-grown coffee plantations only, in forest fragments only, or in both habitat types near San Luis, Costa Rica during 2005-2008.

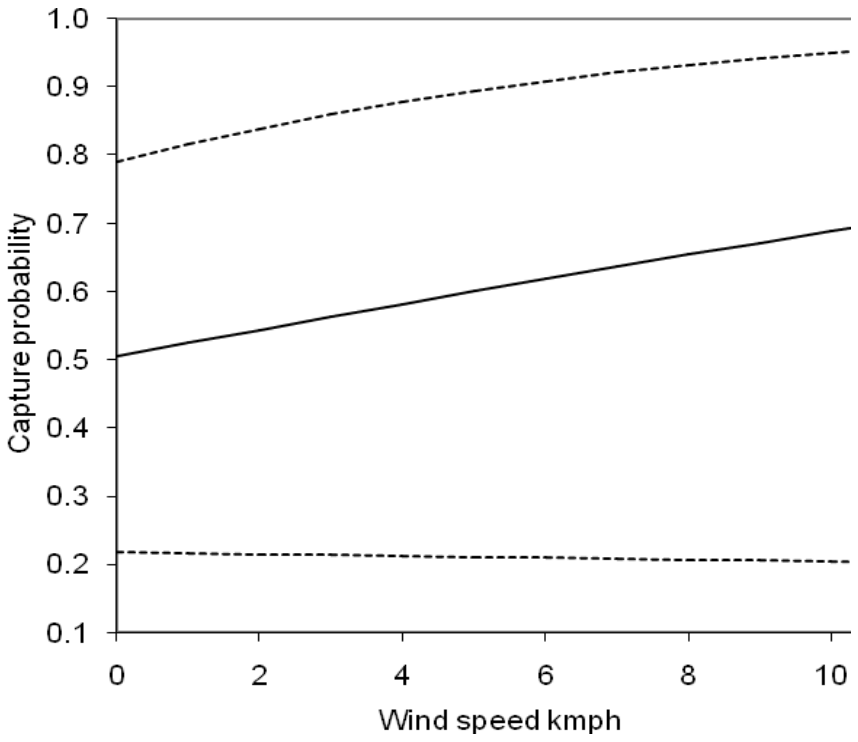
Captured in Coffee sites only	Captured in Forest sites only	Captured in both habitat types
AFLY	BHNT	BCMotmot
BASH	BWOC	BTSL
BBFL	CBT	BWW
BGTAN	ERF	CANW
BORI	KBTU	CCR
BRJA	OBTR	DCFL
CQD	OWOC	ETUC
GKIS	PXEN	GCW
GSAL	RBWR	HWRN
LAWTHR	RCAT	KTYW
LELA	SRTAN	LGNT
LFL	WEW	LTM
MELA		OBFL
MTR		OBNT
MTYR		OSFL
PHV		OVN
RBPS		PWRN
REVI		RCW
STTA		RWOC
WWOC		RWWRN
WWPWEE		STHR
YBEL		SWOC
YBFL		TENW
YCEU		WEGS
YGV		WILW
YMFL		WTDV
YTV		WTHR
		WTR
		YBC
		YFGQ
		YFL
		YTBF
		YTEU
		YTYR

Graph 1. Variation in capture probabilities among seasons (A) and among sampling bouts (B) for avian communities in San Luis, Costa Rica. There was insufficient data to estimate capture probability during the wet, non-peak breeding season. Whiskers represent 95% confidence intervals.

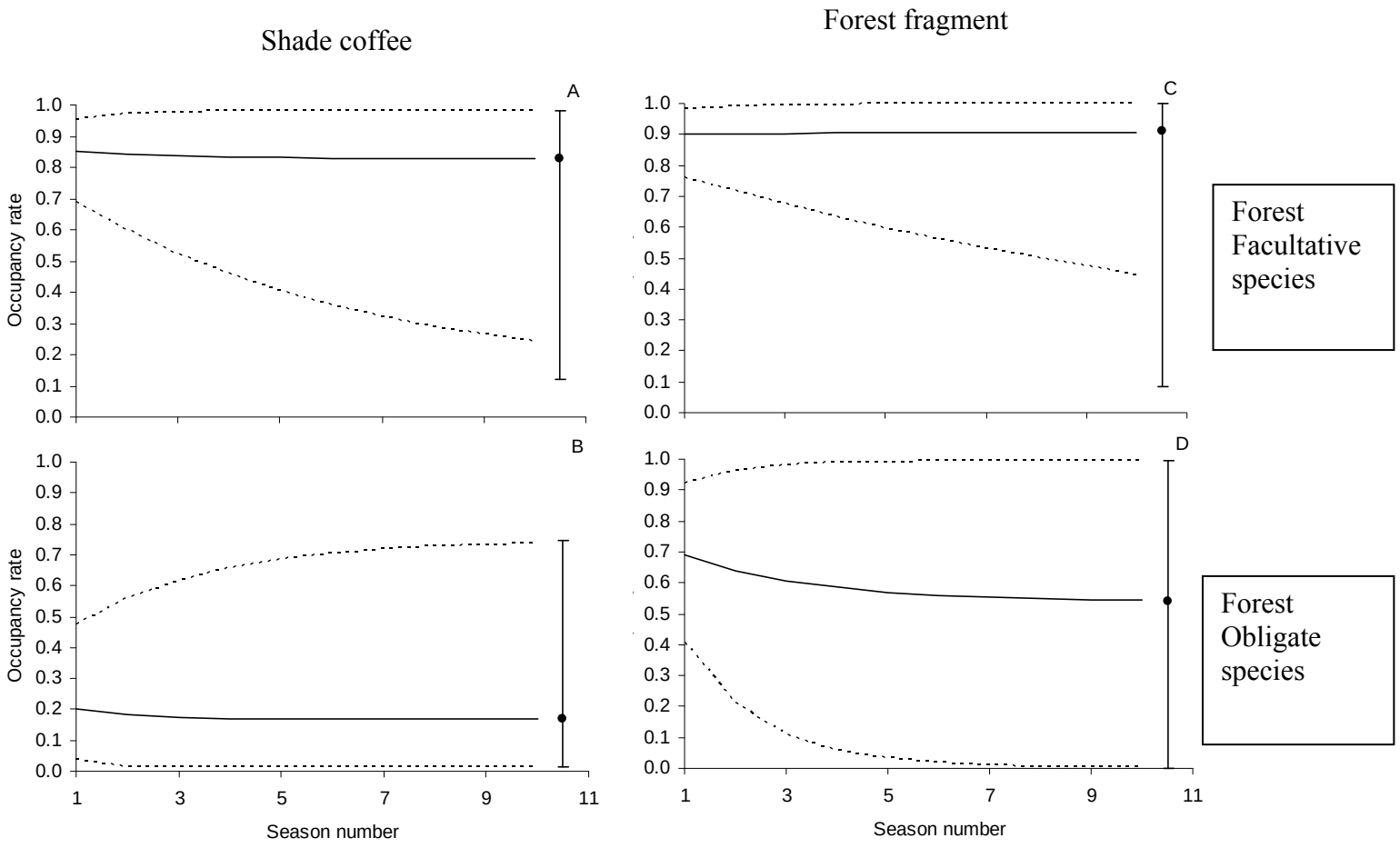




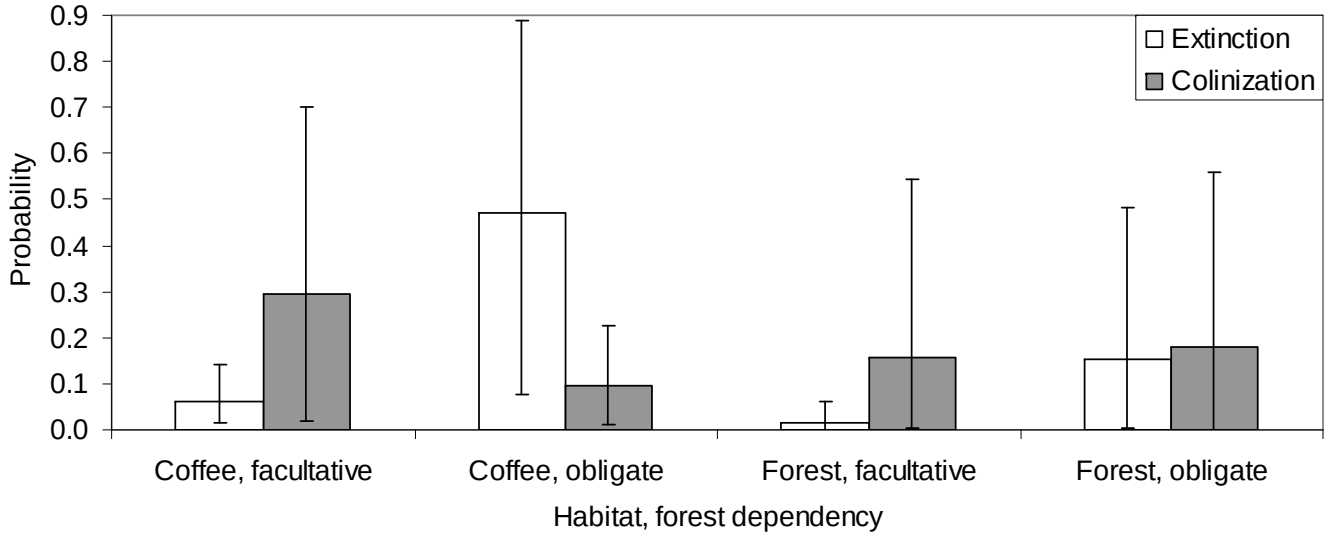
Graph 2. Predicted effect of wind speed (kmph) on capture rates of bird species in shade coffee and forest fragments near San Luis, Costa Rica. Capture rate increases with increasing wind speed. Estimates in graph are based on a model that assumes captures occur during the first sampling bout for the dry, non-peak breeding season. Dashed lines represent 95% confidence limits.



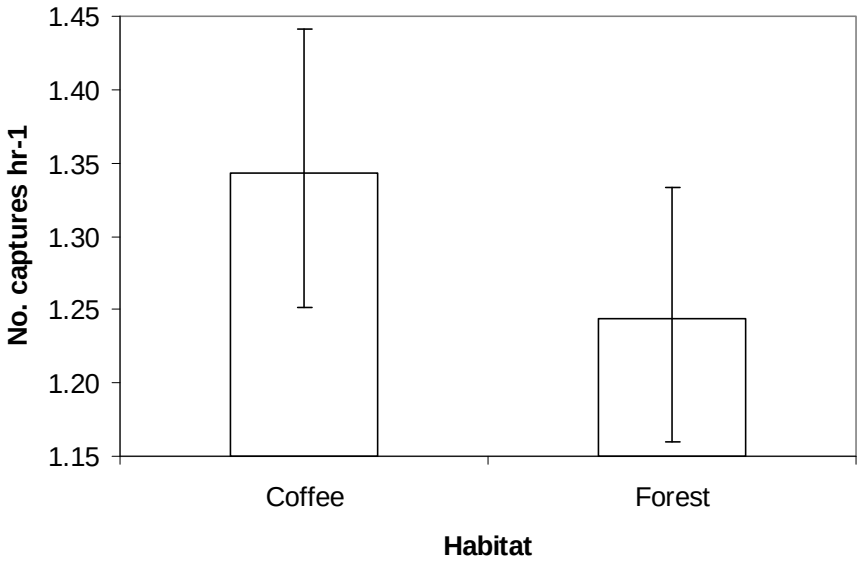
Graph 3. Predicted occupancy rates for avian communities in shade coffee and forest fragments across seasons near San Luis, Costa Rica. Community groupings include the forest facultative guild in shade coffee (A), and in forest (C), and the forest obligate guild in shade coffee (B) and in forest (D). Season 1, which is the leftmost point in each graph, represents the initial occupancy estimate for each guild-habitat combination. Change in occupancy across seasons was based on constant extinction and colonization rates over space (within each habitat), time, and remaining avian guilds. Solid lines represent mean predictions, and dashed lines represent 95% Bayesian credibility intervals.


Shade coffee
Forest fragment
A
B
C
D
Occupancy rate
Season number
Forest Facultative species
Forest Obligate species

Graph 4. Variation in extinction and colonization rates between forest-dependency guilds and between shade coffee and forest fragments near San Luis, Costa Rica. Whiskers represent 95% Bayesian credibility limits.



Graph 5. Total number of individual captures by net hours in two habitat types of San Luis, Costa Rica. This graph illustrates that although the capture per net hour was higher in shade coffee plantations, the confidence intervals overlapped significantly.



Appendix I. Common name, bird code and scientific name of bird species captured during a study of the diseases birds in two habitat types in San Luis, Costa Rica.

Common Name	Code	Scientific Name	Guild Membership
Alder Flycatcher	AFLY	*Empidonax alnorum*	M, F, Om, L
Baltimore Oriole	BORI	*Icterus g. galbula*	M, F, Om, L
Barred Antshrike	BASH	*Thamnophilus doliatus*	R, O, I, L
Barred Woodcreeper	BWOC	*Dendrocolaptes certhia*	R, O, I, L
Black-and-white warbler	BWW	*Mniotilta varia*	M, F, I, L
Black-headed Nightingale thrush	BHNT	*Catharus mexicanus*	R, F, Om, L
Blue-crowned Motmot	BCMotmot	*Momotus momota*	R, F, Om, L
Blue-gray Tanager	BGTAN	*Thraupis episcopus*	R, F, Om, H
Boat-billed flycatcher	BBFL	*Megarhyncus pitangua*	R, F, Om, H
Brown Jay	BRJA	*Cyanocorax morio*	R, F, Om, H
Buff-throated Saltator	BTSL	*Saltator maximus*	R, F, Om, L
Canada Warbler	CANW	*Wilsonia canadensis*	M, F, I,H
Chiriqui Quail-Dove	CQD	*Geotrygon costaricensis*	R, O, Om, L
Clay colored robin	CCR	*Turdus grayi*	R, F, Om, L
Common Bush-Tanager	CBT	*Chlorospingus opthalmicus*	R, O, Om, L
Dusky-capped Flycatcher	DCFL	*Myiarchus tuberculifer*	R, F, Om, L
Emerald Toucanet	ETUC	*Aulacorhyncus prasinus*	R, F, Om, H
Eye-ringed Flatbill	ERF	*Rhynchocyclus brevirostris*	R, O, Om, L
Golden-crowned warbler	GCW	*Basileuterus culicivorus*	R, O, Om, L
Grayish Saltator	GSAL	*Saltator coerulescens*	R, F, Om, L
Great Kiskadee	GKIS	*Pitangus sulphuratus*	R, F, I, H
House Wren	HWRN	*Troglodytes aedon*	R, F, I, L
Keel-billed Toucan	KBTU	*Ramphastos swainsonii*	R, F, Om, H
Kentucky Warbler	KTYW	*Oporornis formosus*	M, F, I, L
Least Flycatcher	LFL	*Empidonax minimus*	M, F, Om, L
Lesser Elaenia	LELA	*Elaenia chiriquensis*	R, F, Om, H
Lesser Greenlet	LGNT	*Hylophilus decurtatus*	R, F, Om, H
Long-tailed Manakin	LTM	*Chiroxiphia linearis*	R, F, Fr, L
Louisiana Waterthrush	LAWTHR	*Seiurus motacilla*	M, F, I, L
Mistletoe Tyrannulet	MTYR	*Zimmerius vilissimus*	R, F, Om, H
Mountain Elaenia	MELA	*Elaenia frantzii*	R, F, Om, H
Mountain Robin	MTR	*Turdus plebejus*	R, F, Om, L
Ochre-bellied flycatcher	OBFL	*Mionectes oleagineus*	R, F, Om, L
Olivaceous Woodcreeper	OWOC	*Sittasomus griseicapillus*	R, O, I, L
Olive-striped Flycatcher	OSFL	*Mionectes olivaceus*	R, F, Om, L
Orange-bellied trogon	OBTR	*Trogon aurantiiventris*	R, O, Om, H
Orange-billed Nightingale-Thrush	OBNT	*Catharus aurantiirostris*	R, F, Om, L

Ovenbird	OVN	*Seiurus aurocapillus*	M, F, I, L
Philadelphia Vireo	PHV	*Vireo philadelphicus*	M, F, Om, H
Plain Wren	PWRN	*Thryothorus modestus*	R, F, I, L
Plain Xenops	PXEN	*Xenops minutus*	R, F, I, H
Red-crowned Ant-tanager	RCAT	*Habia rubica*	R, O, Om, L
Red-eyed Vireo	REVI	*Vireo olivaceus*	M, F, Om, L
Roufus-breasted wren	RBWR	*Thryothorus rutilus*	R, F, I, L
Ruddy Woodcreeper	RWOC	*Dendrocincla homochroa*	R, F, I, H
Rufous-browed Peppershrike	RBPS	*Cyclarhis gujanensis*	R, F, I, L
Rufous-capped Warbler	RCW	*Basileuterus rufifrons*	R, F, Om, L
Rufus-and-white Wren	RWWRN	*Thryothorus rufalbus*	R, F, I, L
Scarlet-rumped Tanager	SRTAN	*Ramphocelus passerinii*	R, F, Om, L
Silver-throated tanager	STTA	*Tangara icterocephala*	R, F, Om, H
Streaked-headed Woodcreeper	SWOC	*Lepidocolaptes souleyetii*	R, F, I, H
Swainson's Thrush	STHR	*Catharus ustulatus*	M, F, Om, L
Tennessee Warbler	TENW	*Vermivora peregrina*	M, F, Om, H
Wedge-billed Woodcreeper	WWOC	*Glyphorhynchus spirurus*	R, O, I, L
Western Wood-Pewee	WWPWEE	*Contopus virens*	M, F, I, L
White-eared Ground-Sparrow	WEGS	*Melozone leucotis*	R, F, Om, L
White-throated Robin	WTR	*Turdus assimilis*	R, F, Om, L
White-tipped Dove	WTDV	*Leptotila verreauxi*	R, F, Om, L
Wilson's warbler	WILW	*Wilsonia pusilla*	M, F, I, H
Wood Thrush	WTHR	*Hylocichla mustelina*	M, F, Om, L
Worm-eating Warbler	WEW	*Helmitheros vermivorus*	M, O, I, L
Yellow bellied flycatcher	YBFL	*Empidonax flaviventris*	M, F, I, H
Yellow Tyrannulet	YTYR	*Capsiempis flaveola*	R, F, Om, L
Yellow-bellied Elaenia	YBEL	*Elaenia flavogaster*	R, F, Om, H
Yellow-billed Cacique	YBC	*Amblycercus holosericeus*	R, F, Om, L
Yellow-crowned Euphonia	YCEU	*Euphonia luteicapilla*	R, F, Om, H
Yellow-faced Grassquit	YFGQ	*Tiaris olivacea*	R, F, Om, L
Yellow-green vireo	YGV	*Vireo flavoviridis*	R, F, Om, H
Yellowish Flycatcher	YFL	*Empidonax flavescens*	R, F, Om, H
Yellow-Margined Flycatcher	YMFL	*Tolmomyias assimilis*	R, O, Om, L
Yellow-throated Brush finch	YTBF	*Atlapetes gutturalis*	R, F, Om, L
Yellow-throated Euphonia	YTEU	*Euphonia hirundinacea*	R, F, Om, H
Yellow-throated Vireo	YTV	*Vireo flavifrons*	M, F, I, H

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CONCLUSIONS

Forest surrogate environments, such as shade-grown coffee plantations provide suitable habitat, which maintains the species richness and abundance of wild birds. However, they are human altered systems which may pose a potential risk to wild birds through exposure to highly mobile backyard chickens and their pathogens. In accord with Friend et al, we recognize the need to focus more attention to disease issues as direct and indirect causes of declining avian populations, from an ecological perspective. Unfortunately, disease investigations in wild birds are often only undertaken following a massive mortality event, or on highly endangered species and they typically focus on mortality alone, ignoring subtle, sublethal or indirect effects caused by one, or a combination of diseases. Although, to our knowledge, no visible mortality events of wild birds has occurred as a result of the introduction of backyard chickens in forest surrogate habitats, we predicted that if wild birds become infected with the aforementioned diseases, there are likely fitness trade-offs to individuals associated with infection and immune defense against viruses and parasite loads, which may translate to effects on population dynamics through indirect and sublethal effects. Additionally, free range chickens can serve as reservoirs of antimicrobial resistance bacteria, which could be disseminated to birds utilizing shade coffee plantations. Recognizing the need for creating more available habitat for avian conservation, further sustainable agroforestry incentives, such as shade-grown cacao, are the wave of the future.

Thus, studies understanding the disease dynamics of wild birds inhabiting these forest surrogate habitats to determine their significance as foci of risk for diseases will motivate policy changes for conservation organizations.

Regarding antimicrobial resistance patterns of avian fecal flora, my results support our expectations that bacterial genera has a strong influence on the level and type of antibiotic resistance and that the isolates from the sites with the highest degree of human disturbance displayed significantly higher probability of resistance. Interestingly, there was no statistical difference in the resistance frequency or prevalence of isolates from birds in sustainable agricultural sites (shade coffee plantations) and forest sites, suggesting that the management of these plantations maintains the integrity of forested habitat in ways not previously explored. Phenotypic and genotypic antimicrobial resistance is a complex issue that is affected by a variety of factors, including species, health status of individual, age, diet, animal production type, bacterial strain, sample and laboratory methodology, geographic location, antimicrobial use, etc. However, the results I obtained do suggest that human persistence (and its associated activities) in these habitats is positively related to higher prevalence of antimicrobial resistance in wild birds that share these habitats.

With regards to avian pathogen prevalence and diversity, generally, we disproved our hypotheses. In the case of some pathogens, temporal scale, sample size, or testing logistics have limited our ability to explore our theory as completely as possible. However, in cases where this was not a limitation, it appears that our suggested mechanisms (the introduction of chickens, the artificial concentration of wild birds, etc) have not played a role in changing the disease dynamics sufficiently to increase the

prevalence and diversity of pathogens in avian communities living in shade-grown coffee. As mentioned before, at least two groups of pathogens deserve further attention: *Hemoproteus* and the diversity and identify of some parasites. One alternative explanation is related to trade-offs—these plantations offer birds superb, constant food subsidies in the form of, for example, crops, planted fruiting trees and a variety of arthropods associated with crops and it has been suggested that shade coffee might act as refuges in times of low food availability in nearby fragments. In addition, preliminary studies indicate that foraging behavior of birds in shade coffee changes significantly. One of the landmark features of Neotropical birds in Monteverde is their participation in mixed species flocks, in which a variety of unrelated species feed and travels together. This behavior is logically thought to be related to predator avoidance. However, there is much less flocking behavior in birds in shade-grown coffee plantations. I have personally observed species which are known to participate in flocking behavior in forested habitat, feeding solitarily on a regular basis and at least one study has come to the same conclusion. Logically, coffee plantations are more "open" than forest and perhaps this allows single birds to monitor for predators more easily without the aid of other birds. Additionally, birds seem to be more homogenously distributed throughout coffee parcels, when compared to forest fragments, and perhaps the entire parcel can be viewed as "one large feeding flock". If that is the case, two significant stressors (food availability and concern over predators) might be lessened for birds that inhabit coffee plantations, allowing energetic resources to be shifted towards immunity. Measuring stress and its effect on an individual or a population is the Holy Grail for most wildlife disease investigators. The level of coarseness of our health parameters may not be able to detect

these subtle differences. However, procedures which could quantify avian immunity in small birds, with relatively non-invasive methods are in development and may be the next wave in determining fine habitat type differences.

Regarding avian community composition, I found that although shade-coffee harbors higher apparent species richness than nearby forest fragments, details about the species composition are important. Indeed, the overall trends we found imply that forest obligate species colonize shade coffee at lower rates than forest fragments, and do not persist in coffee to the same extent as in forest fragments. Forest obligate species had lower interseasonal persistence in shade coffee plantations than in unfarmed forest fragments. Thus, mixed conservation strategies that utilize shade coffee not as an absolute forest surrogate habitat, but rather as complimentary to connect and buffer remaining forest fragments are recommended.