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The epidemiology of avian mycobacteriosis: Using social network analysis to uncover patterns of disease transmission

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Public Health (Epidemiology)

by

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2018

DEDICATION

In memory of my parents, whose eternal encouragement inspired this journey;
and to my husband, whose love and patience made it a reality.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AFB	Acid-fast bacilli
CI	Confidence Interval
DNA	Deoxyribonucleic Acid
ERGM	Exponential Random Graph Model
GI	Gastrointestinal
HIV	Human Immunodeficiency Virus
IQR	Interquartile range
IV	Intravenous
MAA	*Mycobacterium avium avium*
NTM	Non-tuberculous Mycobacteria
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RFLP	Restriction Length Fragment Polymorphism
RR	Relative Risk
SARS	Severe Acute Respiratory Syndrome
SDZG	San Diego Zoo Global
SNP	Single Nucleotide Polymorphism
SD	Standard Deviation
TB	Tuberculosis
WGS	Whole-genome Sequencing

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ABSTRACT OF THE DISSERTATION

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Using social network analysis to uncover patterns of disease transmission

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Background: Transmission of avian mycobacteriosis is generally considered a contagious process, but is not well understood and environmental sources may be important. The large, dynamic population of birds at San Diego Zoo Global (SDZG) with complete population ascertainment over a 22-year period offered an opportunity to use social network analysis to understand disease epidemiology and test for patterns of contagion.

Objectives: Study one evaluated the social network structure of birds for evidence of a contagious process. Study two examined patterns of genetic similarities using whole genome sequencing (WGS) along pathways of network connectivity. Study three examined whether network connectivity predicts future disease.

Methods: Study one identified cases of mycobacteriosis and constructed a social network from enclosure histories (n=16,430) in the SDZG population. Stratification of network edges by spatial and temporal characteristics tested for contagion and other drivers of disease in directly- and indirectly-connected birds. Study two characterized mycobacteria isolated from 124/275 cases. For the subset with WGS (n=97), the probability of having similar genotypes given connectivity was estimated and significance determined from random permutation tests. Study three used longitudinal, mixed-effects logistic regression to evaluate the association between network exposure and mycobacteriosis development.

Results: Study one: Disease clustered significantly among directly- and indirectly-connected birds. The RR of disease given exposure to 2° contacts never housed in the same enclosure was 1.31 (p=0.004), providing strong evidence that a contagious process is present, because the association persisted when common environmental exposure was removed. Study two: *Mycobacterium avium avium* (MAA) and *M. genavense* were the most common species. The WGS showed genotypes of MAA were significantly related along paths of network connectivity; however, no significant patterns were identified for *M. genavense*. Study three: Results showed significant associations between direct (OR=2.15) and indirect (OR=1.56) exposure to positive birds (compared to no exposure) and mycobacteriosis. Risk-stratified models provided estimates with further characterization of exposure; not all findings were robust to model variation.

Conclusion: Social network analysis was a powerful method for evaluating complex contact patterns and mycobacteriosis. The data strongly support a contagious process, show low transmissibility, and provide new information on disease epidemiology.

CHAPTER 1: INTRODUCTION

PART 1: AVIAN MYCOBACTERIOSIS AND DISEASE TRANSMISSION

One of the biggest challenges in zoo-managed bird populations is implementing effective disease management protocols for the well-known bacterial disease, avian mycobacteriosis. This disease has long been thought of as highly contagious, readily passed from bird-to-bird through the fecal-oral route of infection. Lack of clinical signs and long incubation periods make it nearly impossible to determine whether birds in-contact with an infected bird have become infected. Therefore, all exposed birds are considered highly suspect for being latently-infected carriers (and eventually transmitters) of what is perceived to be a highly-transmissible disease. This has a tremendous negative impact on bird management which may not be reasonable if the assumptions of transmissibility are not well founded.

Mixed-messages frequent the literature regarding the most important sources of infection. Many emphasize the role of other birds in transmitting the disease primarily via the fecal-oral route,[1–3] while others discuss the importance of the environment in harboring mycobacteria.[4–6] A comprehensive review on avian mycobacteriosis published by Tell and colleagues[7] demonstrates some of the confusion surrounding the sources of infection when the authors state, "*As the mycobacteria pathogenic for birds are opportunistic saprophytes, the primary source of infection is a contaminated environment. Faeces from infected birds which are shedding the organisms via the intestinal tract are a principal source of infection for other birds."* At the center of the confusion are the two distinct, but tightly linked conceptualizations of the role of the environment in disease transmission. One scenario implies that infections are opportunistic and due to potentially pathogenic mycobacteria in the environment. The other is that the environment is an intermediary collection place for infectious organisms that are being indirectly transmitted from bird-to-bird. Fundamental to understanding the epidemiology of this disease and identifying the

best control measures is the ability to distinguish between these two major sources of mycobacterial infections: other infected birds versus the environment.

The goal of this review is to 1) describe the history of the discovery of avian mycobacteriosis and early establishment of its epidemiology, 2) summarize what has been reported about transmission, and 3) examine whether the environment could be an important source of infection or an intermediary to bird-to-bird transmission. Resurrecting early literature and synthesizing it with more recent literature forms the appropriate base for new studies of the epidemiology of this disease.

OVERVIEW OF AVIAN MYCOBACTERIOSIS

Avian mycobacteriosis is characterized by histiocytic to granulomatous inflammation observed in single or multiple tissues, but commonly in the intestines, liver, lungs, spleen, and bone marrow.[1,8,9] The inflammatory process slowly leads to organ impairment and eventually to death.[8] The species of Mycobacteria most commonly infecting birds include *Mycobacterium avium* subsp. *avium* (MAA) and *M. genavense.*[9–12] Several other species have been reported as causes of opportunistic infections, such as *M. intracellulare, M. flavescens, M. xenopi,* and *M. fortuitum* and are reviewed elsewhere.[13] The close phylogenetic relationship between MAA (and other avian Mycobacteria) to *M. tuberculosis,* the most common etiologic agent of human tuberculosis (TB), has led to the common name "avian tuberculosis" or "avian TB", implying an infection process similar to that of human TB. Because there are several agents that cause avian mycobacteriosis and all are distinct from the causative agents of human TB, we refer to this tuberculosis-like disease using the more suitable terminology "mycobacteriosis" throughout this review as has been recommended by others.

The disease is slow-progressing with long latent and/or infectious periods suspected to last for months or possibly years.[1,14,15] Most cases are diagnosed postmortem when acid-fast

bacilli are observed through histopathologic examination of fixed tissues. Birds are often not diagnosed clinically due to non-specific clinical signs and lack of sensitive antemortem diagnostic tests for reasons previously reviewed.[7] PCR tests have high analytical sensitivity,[15] but they are of limited value clinically because of the potential for sampling error and false positives due to pass-through of mycobacteria in the feces. When cases are diagnosed clinically, they are difficult to treat and require long-term, multiple antibiotic administration. Results of these treatments are often variable. This is due in part to difficulties in administering drugs to avian patients. It is also due to increasing microbial resistance coupled with decreasing numbers of new antimicrobial drugs in development that are proven efficacious for the treatment of avian mycobacteriosis.[16] Due to these treatment challenges and the perceived zoonotic risk, euthanasia is often the outcome when ante-mortem disease is recognized.

The gold standard for diagnosis is culture of the bacteria from infected tissues using special media. However, culture has relatively low diagnostic sensitivity due to the fastidious nature of the organism,[7] the variability in bacterial numbers in infected tissues, and the potential for sampling error. *M. genavense* requires special techniques, which may not be readily performed by commercial diagnostic laboratories.[17,18] For all of these reasons, diagnosis is often based on histopathology.[1] Molecular assays are also beginning to aid in diagnostic confirmation with the advantages of providing rapid results, detectability when low numbers or organisms are present, and providing species level identification through testing conserved areas of the genome.[13] Testing for divergent areas of the genome can discern between genotypes of the same species of *Mycobacterium* and may be suitable for answering questions on strain sharing related to transmission.[17]

POPULATION SIGNIFICANCE

Poultry

For years, avian mycobacteriosis was recognized as an important disease of farmed poultry that contributed to large-scale economic losses with chronic unthriftiness, decreased production, and mortality.[14] In the early part of the 20th century, it was considered the most widespread and economically important disease of the poultry industry in the United States and prevalent throughout Europe based on several reports from Denmark, the United Kingdom, France, and Germany that have been reviewed elsewhere.[14,19] In the United States, over 162 million chickens from 1.5 million flocks were tested throughout the country during the ten-year period from 1925-1934 and about 5.3% were infected.[20] In the entire Northern Central United States where a large portion of the poultry industry was found, it was estimated that between 50% and 64% of flocks had the disease and the percentage of total chickens infected ranged from 3% to 5.4%.[14] Prevalence was higher in some regions and subgroups of birds. For example, in Minnesota the disease was present in more than 60% of flocks. On certain farms, infected chickens may have reached 75% or more.[21] Feldman[14] cited a personal communication reporting 66/72 flocks in South Dakota (93%) reacted to tuberculin. Over 9,000 chickens were tested and 12.8% of younger birds less than a year were infected, but the prevalence increased to over 24% among chickens greater than 1 year in age.

It was presumed that once the disease was established in a flock it would insidiously spread from bird-to-bird undetected and eradication was near impossible. However, disease control measures put into place in the mid-1900's focused largely on sanitation and rapid turnover of populations and helped greatly reduce prevalence of disease. Such measures included disinfection with cresylic compounds, large-scale replacement of dirt flooring, indoor confinement, all-in/all-out bird movement within houses, raising adults and young separately, and

the shift to maintaining young stock.[1,22] Since this time, the disease has been largely eliminated as a concern for the modern intensive poultry industry.

Wild birds

Generally, mycobacteriosis is not considered a prevalent or population-limiting disease of free-ranging birds. A post-mortem survey of various species of birds in the Netherlands (1975-1985) found only 0.7% (87/11,664) with mycobacteriosis using tissue culture and serology.[23] A recent cross-border study between Austria and Czech Republic reported no cases of avian mycobacteriosis using PCR-based assays in 110 freshly sampled carcasses.[24] Another study from Georgia, USA found only seven cases in 827 necropsied birds (0.8%) between 2006-2011.[25] *Mycobacterium avium avium* and *M. genavense* was found in 4% (2/45) and 18% (3/38) of fecal samples, respectively, collected from free-ranging scarlet macaws in Costa Rica.[26] However, large outbreaks have been reported. Approximately 7% and 10% of Whooper swans and Bewick's swans, respectively, died with mycobateriosis while overwintering at the Wildfowl Trust in Great Britain.[27] A 1999 report[28] on mass mortality of over 18,500 lesser flamingos (*Phoeniconaias minor*) in Kenya identified mycobacteriosis as a contributory cause. The authors suspect the environmental pressures of an intense algal bloom promoted unusually high case-fatality to an already endemic mycobacterial disease.

Pet birds

Infections in pet birds seem to be rare based on large surveys of cases submitted to diagnostic laboratories. In a study of 1961 pet birds of different species in Northern Italy, only 27 (1.4%) were diagnosed with mycobacteriosis based on histopathology and molecular assays.[29] Another survey[11] of 9,241 Psittacines submitted for diagnostics to a California state laboratory over a 27-year period found only 123 cases of mycobacteriosis (1.3%). Many of the birds in this study were pets of private owners, although estimates of infection among just privately-owned

pets were not provided. One recent study,[30] however, showed up to 91% of pet birds within the same flock were infected with *M. genavense*. *Mycobacterium genavense* was the most commonly diagnosed species of mycobacteria in both of these aforementioned studies, and has been reported as being the most common mycobacterial pathogen of Psitticines.[10,18]

Euthanasia is usually recommended for pet birds diagnosed with mycobacteriosis due to the perceived zoonotic risk.[16] People who are immunocompromised due to other diseases (e.g., HIV, cancer treatment), those with other pulmonary diseases or lung damage, the young and the elderly are generally at a higher risk of acquiring non-tuberculous mycobacterial (NTM) infection,[31] but it is unknown whether a pet would be the likely source. Some have reported that avian and human types of mycobacteria are genetically distinct, therefore unlikely to cross transmit.[4,32,33] There is also a general lack of research to develop optimum treatment methods. Treatment of disease has been attempted and approaches have been reviewed;[16] however, it is costly and difficult to administer over a long course, and therefore, is often unsuccessful.

Zoo birds

Avian mycobacteriosis has always been an important disease for zoo populations, even described by pathologists from zoos in London and Hamburg prior to the discovery of the etiologic agent.[34,35] Overall, the reported incidence and prevalence of disease is variable and probably depends on several factors, including the represented species and their susceptibility along with animal husbandry and management. One historic report[36] from 1907 stated that 118/459 (26%) of the birds from the Berlin Zoo were infected. The Philadelphia Zoo reported the prevalence of mycobacteriosis based on 14,255 post-mortem exams with histopathology ranged between 0.5-16%, averaging 5.6% from 1901-1975.[37] The National Zoo reported 46/516 (9%) of birds necropsied between 1969 and 1975 had avian mycobacteriosis. Prevalence was 14% when excluding193 neonatal deaths.[38] Prevalence reported in a wildfowl park in the United Kingdom

was high in the 1980's, with 783/2377 (33%) of adult birds showing evidence of mycobacteriosis based on post-mortem exam that included histopathology.[39] The San Diego Zoo and Safari Park (collectively referred to as San Diego Zoo Global; SDZG) had a low cumulative incidence of infection between 1991-2005, with 1.2% (172/13,972) of all birds affected; 3.7% (172/4604) among birds with complete post-mortem exams that included histopathology.[9]

Among zoo birds, disease management has been based on the assumption of bird-to-bird transmission. There is wide concern that birds exposed to infected birds are considered likely to become subclinical carriers and eventually transmitters of the disease. Fear that subclinical carriers will spread the bacteria to the naïve population in the same aviary has led to elaborate disease mitigation efforts. These include intensive screening, long quarantine periods (recommended >6 months[1]), halted breeding, halted movement in and out of exhibits, environmental clean-up, and depopulation.[1,38,40–43] Some of these recommendations have been borrowed from the poultry industry, where birds are held in high-density pens and have in-door confinement. Even when implemented, however, birds in zoos still acquire disease.[43] Furthermore, for zoo-based conservation programs, these efforts have large negative impact on population breeding, sustainability, and reintroduction efforts.

DISEASE DISCOVERY AND CURRENT CLASSIFICATION

Knowledge of a tuberculosis-like disease in birds was well-established by pathologists in Europe before the causative agent of human TB was discovered. Crisp was one of the first to report a tuberculosis-like disease in chickens and pheasants in England in 1868, and he successfully transmitted disease to healthy chickens through experimental injection of infected material.[44–46] One of the first reports of the disease in zoo birds came from Paulicki in 1872,[35] who noted the occurrence of a spontaneous, chronic disease in 21 pheasants and ducks at the Hamburg Zoo. He described the weight loss, lesions of the air-sacs, and tumor-like formations in

the liver, spleen, intestines, lungs and lymph nodes and noted similarities between the lesions seen in birds and those present in humans.

The ground-breaking discovery of the etiologic agent of human TB by Koch[47] came at a time when human TB had reached epidemic proportions in North America and Europe.[48] Not long after Koch discovered the bacterium and demonstrated its ability to cause TB in humans and cattle, a similar agent was observed in the tissues of birds.[34,49] Along with others, Koch initially thought the bird bacterium was identical to that of human and other mammalian TB,[50] but he later abandoned this idea when he recognized that TB of poultry was different than that of humans and cattle.[51] This latter conclusion was supported by the work of several researchers who conducted experimental studies in the late 1800's to show important distinctions in in-vitro growth, pathogenicity, and transmissibility of the bacilli that distinguished avian from human tuberculosis. Their works have been reviewed extensively by Feldman.[14] The knowledge they obtained in differentiating the etiologic agent of avian from human and bovine TB laid the foundation for broadening the scope and understanding the unique epidemiologic aspects of the disease in birds. Since that time, modern genetics classifies the agents of human tuberculosis (e.g., *M. tuberculosis* and *M. bovis*) and avian mycobacteriosis (MAA, *M. genavense*, and several others) as distinct species and/or subspecies of the genus Mycobacterium.

Three major groupings of mycobacteria are commonly referenced today based on human medicine:[52] 1) *M. tuberculosis* complex which causes human tuberculosis and includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. orygis***,** and *M. canetti*; 2) *M. leprae* and *M. lepromatosis* which cause leprosy; and 3) the nontuberculous mycobacteria (NTM) which include other mycobacteria that cause opportunistic infections in humans. Included in this category are the common avian pathogens: MAA and *M. genavense*, among several others. The major distinguishing feature between the *M. tuberculosis* complex and the NTMs is primary versus

opportunistic pathogenicity for humans. This anthropocentric view does not hold as neatly for animals and it is better to classify organisms as primary versus opportunistic pathogens.[53] Primary pathogenic mycobacteria live and replicate primarily within a host (e.g., *M. tuberculosis*). Some pathogens, such as *M. a. paratuberculosis,* the causative agent of Johne's disease in hoofed mammals, can remain viable in the environment for extended periods of time,[54] but it ultimately needs an animal host to survive. Opportunistic pathogenic mycobacteria are mycobacteria that have adapted to living in environmental biotic communities and will sometimes infect a host given the right situation.[31] The major avian pathogens, including *M. genavense* and MAA, are considered opportunists and have been reported in a wide range of species, including humans.[53,55,56]

WHAT WE KNOW ABOUT DISEASE TRANSMISSION

Perhaps by analogy to the discovery of human tuberculosis, avian mycobacteriosis has always been presumed to be contagious and readily passed between infected and susceptible individuals. For example, in 1883 Ribbert[49] successfully transmitted infection to two chickens through intraperitoneal injection, however he could not transmit it when chickens were given feed mixed with infected feces over several months. Despite lack of evidence, he still concluded that the disease was spread by infected fecal contamination from other birds.

Characteristic lesions in chickens may have also fueled assumptions about disease spread, with thousands of organisms commonly observed in intestines of infected animals.[14] It was reasonable to assume that the presence of such a lesion provided an opportunity for the pathogen to spread to others through fecal contamination. Based on years of experience in histologic examination of different species, we now know that not all birds acquire intestinal lesions and not all lesions are laden with mycobacteria.[9] Distribution and characteristics of lesions in birds seem highly specific to the individual immune response and route of infection. A spectrum of diverse

TB lesions has also been characterized in human hosts and results from dynamic host-pathogen interactions.[57]

Inoculation experiments

Controlled experimental studies are the most definitive method of verifying disease transmission. This is especially true for diseases like mycobacteriosis that have non-specific clinical signs and long incubation and latency periods. The majority of experimental transmission studies date to the early 20th century when there were concerted efforts to understand the basic biology of a disease that was important to the poultry industry. Studies are buried in historic literature, books, and government documents (e.g., Feldman,[14] Van Es and Schalk,[58] and Griffith[59]). The original reports and details of the experimental designs were not always available for rigorous scientific evaluation. Here, we summarize some of the findings relevant to transmission from these early studies, relying heavily on others' assessment of scientific rigor and interpretation of results. Of note, nearly all experimental research has used MAA as the main inoculum, so it is unknown to what extent these principles apply to other important avian pathogens, such as *M. genavense*.

There is no doubt that birds are susceptible to the infectious bacilli when directly administered (i.e., introduced intravenously, intramuscularly, intraperitoneally, subcutaneously) (e.g., Tell *et al*.[60] and Ledwon *et al*.[61]). The subsequent disease will vary with susceptibility of the bird, virulence of strain, dose, route of inoculation, and duration of the experiment.[14] The relevance of direct inoculation to natural transmission, however, is more tenuous. Even experimental infection of chickens following the ingestion of mycobacteria was historically recognized as uncertain; large quantities of bacteria given repeatedly over long periods of time were needed to induce disease.[14,62,63] Infections are not always observed after oral administration. Some early experiments noted that adult chickens appeared to be resistant, withstanding

continuous exposure to the bacteria over a long period without developing lesions.[14,63,64] There is a dose-response relationship, where injection with lower doses results in more chronic, slower-progressing infection.[59,65,66]

Free-contact experiments

While infection through direct administration of an inoculum does establish the infective potential of a disease agent, it does not specifically address important questions of transmissibility and the resulting infection is unlikely to follow a natural timing of disease progression. Questions on the transmissibility are best addressed through experimental studies that mimic free-contact conditions of wild or managed birds with each other or with an infected environment; however, there is a paucity of such information in peer-reviewed literature. Most studies were conducted before 1920 with limited detail and inaccessible for thorough review. Experimental details were unknown in many instances, including how the final disease status of experimental subjects was determined. Fortunately, Feldman[14] provided a more contemporaneous review of a number of these studies.

In general, studies found transmission through free-contact with infected birds and their contaminated environment was low. Experimental studies often showed little to no transmission when healthy chickens were in contact with either diseased birds or their contaminated environments. Van Es and Schalk[58] cite other researchers who failed to observe transmission in contact experiments. Giltner did not observe transmission of the disease to seven hens that were housed in pens with mycobacteriosis-positive birds or housed in their contaminated environments. He also did not observe lesions in healthy hens after they were in contact for five weeks with other hens in the advanced stages of disease. Hastings, Halpin and Beach, found 3/7 hens developed disease after a year of contact exposure to eight diseased birds. Van Es and Olney[67] found low incidence of transmission from experimentally infected birds when adult

chickens were exposed to more sanitary (2.7%; 9/333) and less sanitary (1.9%; 6/313;) conditions. A higher incidence of transmission was observed in young chickens exposed at 8-10 weeks of age compared to the older birds: 14.6% (40/274) of young chickens in the more sanitary conditions developed disease, and a nearly identical proportion (14.4%; 54/374) in the unsanitary conditions developed disease. Shalk *et al*.[63] found lesions in 10/98 (10%) young chickens that lived in a contaminated environment for 4-5 months. Feldman *et al*.[64] found that 25% (7/29) of healthy adult chickens developed mycobacterial lesions after exposure for 20 months to others that had mycobacteriosis. These historic studies support a conclusion that young chickens are more susceptible to infection than adults, but both groups seem relatively resistant.

Further likelihood of infection appears to vary between species, although this could be related to either behavioral gradients in exposure or to susceptibility to infection. Studies on corvids (*Corvus frugilegus*) and domestic geese (*Anser anser f. domestica*) and ducks (*Anas platyrhynchos f. domestic*) documented no evidence of transmission after 238 and 253 days, respectively.[68,69] Pavlas[70] mentioned another study that documented no development of lesions after free contact of ducks to (presumably) other infected birds,[71] but the original report was not accessible for further review.

Beyond the research mentioned above, experimental studies documenting avian mycobacteriosis transmission through free contact that mimics natural conditions could not be found. We speculate that such studies were probably common, but are buried in governmental reports (e.g., Van Es and Schalk[58]) and books such as Feldman's.[14] Several of the studies are written in languages other than English and may not be retrieved using English search terms. Review of early studies highlights the scarcity of information on transmissibility that includes detailed data on experimental design. This makes basic research on experimental transmission of *Mycobacterium* spp. in birds relevant today.

Experimental transmission studies need considerations for design of levels of exposure to infected animals (e.g., inoculation and verification of infection, amount of infectious agent be shed into the environment, and frequency of shedding). Additionally, specifics of disease development in susceptible animals (e.g., the proportion of susceptibles that develop disease, and how long it takes to develop disease at different exposure levels) should be carefully documented. Control populations are always needed, but seem to be lacking in the literature. An example of a control group would be to include groups of birds exposed to the same environment, but with no access to the infected birds used as the experimental treatment. If inferring transmission from other birds, efforts should be made to mitigate exposure to major sources of environmentally-acquired mycobacteria, such as using sterile water sources. Attention should be given to accommodate the expected, long incubation and latency periods to help determine relevant intervals of follow-up for determining disease status. Consideration for differential effects between important bird pathogens, such as MAA and *M. genavense* should be given. Numerous experimental studies on *M. a. paratuberculosis* pathogenesis and transmission in hoofed mammals have documented some of the aforementioned variables, providing good examples of experimental studies for chronic mycobacterial disease of animals. Such studies are referenced elsewhere[72] and similar research among birds would be useful for understanding transmission.

Observational analytic epidemiology

Observational studies of naturally occurring disease are a better reflection of transmission in the real world than to experimental inoculation. However, interpretation can often be difficult due to the inherent biases that exist in observational data. In experimental studies, the investigator has control over factors that affect the disease course, such as the species of birds, the strain of Mycobacterium, dose, route of infection, and duration of the study. In observational studies, there is no control over such factors. Therefore, careful epidemiologic study designs and data analyses

must be implemented to draw firm conclusions and attention should be given to minimize and understand selection and information biases.[73] Investigators need to use carefully selected control comparison groups and consider confounding factors that could cause misinterpretation of the results. In our review of the literature, we did not find many observational epidemiologic studies that address the aforementioned issues. Proportional mortality due to avian mycobacteriosis has been reported[39,74] among birds at The Wildfowl & Wetlands Trust in Slimbridge, UK. Avian mycobacteriosis accounted for 33% (778/2334) of all deaths during a 10-year period from 1980-1989 and 84% (102/121) in the endangered white-winged wood duck (*Cairina scutulata*) from 1976-1991.

We examined patterns of avian mycobacteriosis during a 14-year period in SDZG bird populations.[9,75] This population is large, with greater than 3,000 birds on average at any given time. All birds were monitored daily by keepers and veterinarians and had ongoing documentation of management and health data. If a bird died while in our care, then the Zoo's veterinary pathologists conducted complete postmortem examinations that included histopathology on all tissues. The prevalence in the entire bird population was low at 1.2%, and 3.2% among birds that had died. The incidence rate was 3 cases per 1,000 bird-years at risk. There was no obvious pattern of increase in disease incidence over time or a "tip of the iceberg" effect as is seen with other infectious mycobacterial disease, such as *M. a. paratuberculosis* infection in cattle.[76] Birds affected were scattered in aviaries throughout our facilities and represented many different taxonomic groups, with pigeons and doves in the order Columbiformes having the highest prevalence. In a subsetted case-control study that tightly controlled for species and age of bird, we found that exposure to other positive birds, especially those exhibiting intestinal lesions, increased risk for disease.

Focusing on 2,413 birds limited to just those exposed to other birds with mycobacterial lesions in the intestines, the prevalence was only slightly higher at 3.5% (85/2,413) and incidence was 7.9 cases for every 1000 bird-years at risk (85/10,814 total bird-years at risk).[75] Among birds that died, 8.6% (85/985) had disease. In this cohort, imported birds were more likely to develop disease than those that hatched at our facility as were those exposed at a young age. Factors related to closer, and more prolonged contact with the diseased bird, including exposure in a small aviary, to the same species, and for longer periods of time also increased incidence. However, one of the remarkable estimates from this study was that 91-96% of birds housed in aviaries with another disease-positive bird never developed disease. Thus, our findings were in agreement with historic studies implicating young age at exposure as a risk, but finding that the disease is probably not highly transmissible.

Based on these findings, SDZG changed its approach to disease management. Prior to 2006, if a case was recognized, we would have instituted a variety of disease management approaches that could have included diagnostic screening of the apparently-healthy enclosuremates, long-term enclosure quarantines that affected population breeding, and extensive environmental clean-up that could have involved replacement of all dirt or composite granite flooring of an aviary. After 2006, population-level screening, quarantining, and environmental clean-up efforts were no longer implemented. To-date (about 10 years later), the incidence and prevalence of disease in the Zoo's population remains similar, with no increasing trends over the time of management change (C. Witte, *unpublished data*).

Molecular epidemiology

Ultimately, studies in molecular epidemiology are needed to determine whether the environment is a primary source or secondary fomite for disease transmission from other birds. Molecular epidemiology connects agent strain types to more precisely characterize the distribution and determinants of disease. Molecular epidemiology has been used extensively to reveal transmission patterns for other mycobacterial diseases, including human tuberculosis (e.g., Walker *et al*.[77] and Gardy *et al*.[78]), *M. a. paratuberculosis* infections in livestock (e.g., Ronai *et al*.[79]), and *M. bovis* transmission from wildlife to cattle (e.g., Biek *et al*.[80]). A thorough review of molecular epidemiology of mycobacteriosis in wildlife has been published elsewhere.[53] Here we review a few studies with data that touch on transmission of mycobacteriosis in birds.

A large genetic study on avian mycobacteriosis used next generation sequencing to compare isolates from 105 birds at SDZG between 1992 and 2015.[12] About 66% (71/105) of these birds could be grouped with at least one other bird based on genetic similarities for a total of 19 different closely related genetic clusters of MAA and *M. genavense*. Among the many diverse genotypes characterized in these birds were seven uncommonly reported mycobacterial species. Some birds had multiple genotypes and a few had multiple species of Mycobacterium. Limitations on our interpretations come from lack of knowledge on within- and between-host evolution of the agent, as well as knowledge of genetic similarities of mycobacteria freely living in the environment, which complicates epidemiologic inferences from genetic data. Linking genetic similarities with specific epidemiologic relationships were not done in this study due to data complexities, but may provide more insight on transmission pathways or lack thereof.

One approach in molecular epidemiology is to determine if the birds from the same outbreak share the same mycobacterial strains. Most studies have found the presence of multiple different strains within a single outbreak, but with several birds sharing some of the strains. Kauppinen *et al*.[81] used molecular DNA fingerprinting to investigate an outbreak of

mycobacteriosis in farmed lesser white-fronted geese (*Anser erythropus*) from a single population during a 3-year period (1992-1994). Four distinct strains of MAA were identified in the 9 cases revealing that the outbreak was not all from a single point-source. Dvorska et al.[82] also found several different species and subtypes of mycobacteria among isolates from a single outbreak of mycobacteriosis in 38 captive water birds. Shitaye *et al*.[83] found different types of MAA using the IS901 virulence marker and RFLP analysis in a single population of naturally infected chickens. Moravkova *et al*.[84] also examined RFLP patterns for MAA IS901 virulence markers in four flocks of infected pheasants. The distribution of virulence markers was tightly linked to the individual flock, but RFLP patterns varied within a flock. At SDZG, we used DNA fingerprinting methods to investigate 41 mycobacteria isolates from case clusters in 18 different aviaries.[13] Two birds that were housed in the same aviary had mycobacteria with the same banding patterns, however most strains were not related, even when the birds were from the same aviary... Another study[85] reported identical isolates of *M. intracellulare* from 7 penguins housed in the same zoo enclosure over a 4 year period; however the true strain-sharing status of the birds is inconclusive. Genetic comparisons were made with the conserved internal transcribed spacer region of the genome that mostly distinguishes between species of Mycobacterium,[86–88] rather than a variable region that can distinguish between different genotypes of the same species.

INFECTIOUS DISEASE STATES

A major challenge in uncovering the epidemiology of avian mycobacteriosis is the chronic nature of infection and disease with its long and variable incubation and infectious (shedding) periods. This makes it difficult or impossible to determine when exposure leading to new infection occurred. The disease could also be spread indirectly, which adds another obstacle for capturing relevant contact that could result in transmission; birds do not have to contact each

other to acquire infection from each other. In this section it is assumed that the infection is transmissible between birds and further describe what is known about the different disease states.

Latent period

The latent period is the time from initial infectious until shedding begins.[73] For avian mycobacteriosis, the latent period is unknown. An experimental bird model administered high doses of mycobacteria intravenously and first detected the agent in feces between 28 and 63 days post-inoculation.[60] If we assume fecal culture is a sensitive method for measuring the onset of shedding (however, this may not be a reasonable assumption; see Tell et al.[89] and Haridy[90]), then the minimum latent period could be around 1 to 2 months. It would presumably be longer under natural conditions that have not been optimized to induce infection. Other factors that may affect a latent period would be the virulence of the strain, the infectious dose, the site of infection, and host level factors that contribute to individual and species susceptibility to disease.

The latent period is important to disease epidemiology to enable identifying the initial exposure that led to infection. For avian mycobacteriosis, the latent period is shorter than the incubation period (further described below). This means that birds could shed and transmit disease without it being detected. This is a characteristic complicates the intervention strategies of diseases.[73]

Incubation period

The incubation period is the time point from which a bird initially becomes infected, to which clinical disease manifests.[73] Some birds never develop clinical signs and others will develop signs very late in the course of disease – within a few weeks or days of succumbing to the infection (M. Sutherland-Smith, personal communication). This may result from a combination of the virulence of the agent, characteristics of the underlying disease, and the host's evolutionary ability to mask disease. Thus, the incubation period is probably long and covers the bulk of the time course of disease development.

Direct inoculation methods are efficient for studying the course of disease with optimized doses for inducing a disease response, and therefore, certainly truncate the incubation time that would be observed under natural transmission conditions. In an experimental study[60] using intravenous (IV) MAA inoculation of Japanese quail, 7 of 8 birds died naturally, with a mean survival time of 68 days (range: 50-86 days post-inoculation). Clinical signs were noted 49-91 post-inoculation. Van Es and Martin[91] demonstrated lesions 12-90 days post-inoculation in a large study of 218 chickens. Dose and response was extensively studied by Griffith.[59] The average lifespan of chickens given 1.0 mg of pure mycobacterial culture was 33 days. Chickens injected with extremely small doses (up to 10 million times less) were killed at 94 days and chronic lesions were documented. Saenz[65] had similar findings; large doses of IV inoculum caused acute, rapid disease and death in 14 to 21 days. Extremely small doses produced a chronic infection that caused animals to die in 5 to 7 months.

Experiments that mimic natural transmission may provide a better indication of incubation times. Some of the historic transmission studies were unavailable for full review, but have been reported by another.[14] In one study, lesions developed within 4-5 months in a small number of the experimental chickens after exposure to a contaminated environment.[63] Bornstedt and Rohrer[92] exposed healthy chickens to infected ones via sharing pens and concluded that 6-12 months were required for disease to become evident. This time frame for natural incubation of mycobacteriosis in chickens was similar to that observed by others.[62] Hinshaw and Bushnell[93] studied seasonal distribution of cases and found that more birds were submitted in summer. They speculated that the chickens likely became infected the previous spring after they hatched and it took 10-14 months to show clinical signs and die. Free-contact experiments by Hejlicek and Treml[94–97] showed evidence of infection around 6-8 months after contact. In pigeons, histopathologic changes were not observed until 380 days post-contact,[98] but positive fecal

culture results were obtained as early as 37 days for poultry[99] and 68 days for pigeons.[98] The youngest case we identified in birds that hatched at SDZG was a 6 month old, Northern red-billed pigeon (*Columba flavirostris flavirostris),*[9] placing a minimum incubation period around 6 months if the bird became infected near its time of hatch. This is similar to those reported in the early studies listed above.

The distribution of a maximum incubation period is more difficult to assess. It is likely longer than the 12 months identified above because experiments are designed to shorten the natural course of disease for experimental efficiency.[60] Fulton and Sanchez reported[1] that birds can die within a few months or live for many. Others[14] have suggested it may take years for a subclinical infection to result in death; however, we did not identify specific evidence documenting an incubation period of several years. The idea that an animal could be infected for years may be based more on the disease course of mycobacterial infections in other species that have recognized long latent stages, such as human TB and Johne's disease in livestock.

Infectious period and degree of shedding

The infectious period is the time during which a bird can transmit the bacteria to another bird and is often referred to as the shedding period. Information on how long a bird may shed the microorganism is important for determining the potential for disease spread. Shedding depends on the location of the lesions within a bird and is probably most relevant if the digestive system is affected due to the tremendous numbers of bacilli that can be exuded from ulcerative intestinal lesions in poultry.[1] Early experimental studies confirmed that chickens inoculated with MAA can excrete the organism in their feces. In one study, MAA was isolated from the feces of all 12 birds in the treatment group and none in the control group following subcutaneous inoculation of chickens.[100] Some of the birds in the group had mycobacteria identified in feces before any clinical signs were present. In a more recent study,[60] fecal shedding was detected as early as 28

days post inoculation in Japanese quail that were injected intravenously with a large amount of MAA. Most birds had positive feces between 42 and 63 days post inoculation. Mycobacteria were cultured from feces at least 3 times in each of the 8 birds during the course of the 91-day study, but only about half (53%; 69/130) of all fecal samples yielded positive culture, suggesting intermittent shedding or shedding of low numbers of organisms on some days. As the disease in the quail progressed, more fecal samples were positive on more days and the number of acid-fast colonies increased using special stains on fecal cytology. These findings demonstrate that birds in more advanced stages of disease shed more organisms with increasing frequency than those in early stages of disease.

MYCOBACTERIOSIS AND THE ENVIRONMENT

The question remains as to whether and to what extent the environment is a primary source of mycobacterial infections in birds. This concept goes beyond considering that the environment is a catching place for fecal contamination leading to indirect disease transmission and implies that environment itself is a source of free-living mycobacterial opportunists.

Mycobacteria in the environment

The increasing importance of NTM infections in humans as a global health concern has greatly expanded the scientific work on the distribution and abundance of NTM in a number of studies in different types of environments. Non-tuberculous mycobacteria are natural inhabitants of water and soil and are in-contact with humans and animals every day.[5] Their hydrophobic properties cause them to adhere to pipes and congregate in biofilms through which they enter the common water supply.[6] They are also present in soil, common in the pine boreal forests,[101] and in acidic soils and swamps in the Southeastern United States.[102] In his 2016 review[31], Falkinham III summarized major sources of NTM mycobacteria to be: peat-rich soils and drainage water from them, U.S. coastal swamp soils, sediment, natural water bodies, drinking water distribution

systems, plumbing of hospitals and homes, instruments with water reservoirs (e.g., humidifiers); refrigerator water, ice, shower aerosols, spas and hot tubs, and biofilms. These findings include mycobacterial species that infect birds. MAA can exhibit a wide variety of behaviors that allow them to freely live in the environment, including extracellular replication, survival or replication in protozoa,[103,104] anaerobic survival and growth,[105] and the formation of biofilms.[106] *M. intracellulare* also readily collects in biofilms.[103,106] *M. genavense* has been found in water supplies.[107]

Infections in non-avian species

It is widely accepted that infections in humans caused by NTM are opportunistic and originate from an environmental source rather than other infected humans. Cases of NTM in human patients have been traced to specific environmental sources in several studies, including a hospital water supply,[108] biofilm in a showerhead,[109,110] general household plumbing,[109] hot tubs,[111,112] an injection device inappropriately cleaned with tap water,[113] a water reservoir used in surgical procedures,[114] and potting soil.[115] Spatial clustering of NTM cases in humans has been linked to environmental and sociodemographic exposures rather than transmission between people (e.g., Maekawa *et al*.[116] and Adjeman *et al*.[117]). The more recent exception to this may be evidence of *M. abscessus* transmission between patients with cystic fibrosis,[118,119] but additional studies are needed to further document transmission potential.

Mycobacterial infections in other animals with NTM are often considered opportunistic and of environmental origin. Tree kangaroos are especially susceptible to mycobacteriosis.[120,121] In a study of a colony of 33 Matschie's tree kangaroos, DNA fragments differed between clinical and necropsy isolates of *M. avium* complex isolates providing no evidence of transmission between animals.[122] Mycobacterial infections in pigs due to *M. a.* complex, including *M. intracellulare* and *M. a. hominissuis* are well-recognized. DNA strain typing methods have been

used to track sources of infection in several studies and most have concluded that environmental sources, such as sawdust and peat, are likely.[123–126] An outbreak of MAA infections in 21 macaques infected with simian immunodeficiency virus (SIV) at the New England Primate Research Center was attributed to drinking water. Different mycobacterial agents were isolated from different macaques based on distinct DNA fingerprint patterns. Some of the patterns specifically matched mycobacterial isolates obtained from water sources used for drinking.[127] Additional examples of NTM species infecting animals has been comprehensively summarized in other reviews.[53,128] In most instances, apart from birds, NTM are considered rare, opportunistic infections of environmental origin.

Infections in birds

The role of the environment established for other NTM infections is probably important for avian mycobacteriosis, but has been largely overlooked. Birds are no exception to the abundance of exposure to environmental mycobacteria. One study that examined 491 environmental samples in an aviary that housed captive waterfowl obtained 24 isolates of MAA and 13 of *M. a. hominissuis* from a variety of compartments, including regurgitated food, mixtures of soil and feces, lake water and sediment, soil and leaves, feed, sand, and invertebrates.[82] In an unpublished study at SDZG, we tested 252 soil samples and 47 water samples collected from 238 aviaries for MAA containing the IS901 virulence marker. We also tested 93 food items that included earthworms, mealworms, and crickets. In total, 68% of soil samples, 53% of water samples, and 38% of food items contained IS901, demonstrating widespread exposure to mycobacteria in our zoo. Samples originating from misters, tap water lines and food items from a warehouse were likely from a non-bird, environmental source (S. Anthony, C.Witte and B.Rideout, unpublished data).

Linking specific avian cases to an environmental source is complicated. Source tracking of human cases was originally done using DNA fingerprinting, which provides enough discriminatory power to determine that a match between the environment and a patient isolate is highly significant and can be used to identify sources of opportunistic infection by environmental mycobacteria.[129] Such methods have been used to strain-type and compare bird isolates with the environment and with each other (e.g., Dvorska *et al.,*[82] Kauppinen *et al*.,[81] and Schrenzel *et al*.[13]), but due to fecal shedding by infected birds it is difficult to determine whether the identified mycobacteria are truly environmental in origin or whether they resulted from contamination by feces of another bird. Some mycobacteria can stay viable in the environment for long periods of time. Schalk *et al*.[63] found viable bacteria in carcasses after being buried at 3 feet for 27 months. The same researchers found infected litter in a barnyard to have virulent avian strains after 4 years. Friend[2] summarizes more findings from environmental studies that show the bacterium can survive long periods in organic substrates. Distinguishing between a non-bird environmental source and indirect transmission is a key question for understanding the epidemiology of this disease.

SUMMARY OF PART 1

In this review, we have focused on aspects of avian mycobacteriosis that have shaped the perception of the disease throughout history. Much of the currently accepted tenets of disease epidemiology seem less grounded in science and more based on perception. Below are the key concepts with support across scientific studies that add to our current understanding of avian mycobacterioisis.

Avian mycobacteriosis is not a highly transmissible disease. This idea is well-supported by historic experimental transmission studies showing that transmission is inefficient when simulating fecal-oral or free-contact transmission. Epidemiologic studies also do not

support epidemic disease spread or a "tip-of-the-iceberg" effect[76] for mycobacterial diseases of birds caused by MAA, *M. genavense*, or other NTM. The severe disease outbreaks reported in the poultry industry in the early 20th century may have resulted from differences in animal management and hygiene practices and therefore do not reflect current disease dynamics. The large die-off of flamingos,[28] which was partially attributed to mycobacteriosis, had other precipitating factors that were thought to be drivers of the mortality event.

Avian mycobacteriosis is a disease that can be caused by different agents. The most common pathogens include MAA and *M. genavense*, but numerous other species of mycobacteria have been reported in birds and have been reviewed elsewhere[53]. Infection caused by the different agents should be considered distinct diseases with the potential for different disease epidemiology and types of transmission. For example, in one study, MAA isolates tended to be far apart genetically, whereas *M. genavense* isolates were much closer related, perhaps suggesting that M. genevense is more directly transmissible.[12]

The environment alone may be an important source of mycobacteria. The most common pathogens of birds, MAA and *M. genavense*, are opportunistic pathogens of humans and animals that freely live and replicate in the environment.[5] A direct role for the environment as a source in at least some avian cases is consistent with what is seen in other species. Patterns in the molecular epidemiology studies reviewed here reveal that different mycobacteria can be associated with single outbreaks,[12,13,81–83] which suggests at least some purely environmental sources may be involved.

Low transmissibility provides new management options. Synthesized data from historic studies and recent literature suggest the disease is not highly transmissible. The perception of the disease based on reports from poultry outbreaks in the early 1900's may not capture current dynamics or sanitation and management practices. The often recommended

management practices of placing enclosures under quarantine while birds are repeatedly screened for infection may not be ideal in zoos. This restricts important conservation and breeding programs. Euthanasia of healthy birds based on having a single positive fecal or based on exposure to an infected bird, can result in significant loss of valuable genetic diversity.

PART 2: ANALYZING SOCIAL NETWORKS IN EPIDEMIOLOGY

The study of networks in epidemiology evaluates how differences in relationships of who we know or who we spend time with drive patterns of disease emergence and health. Klovdahl[130] conducted one of the first epidemiologic studies using a network approach. He evaluated the hypothesis that an infectious agent caused AIDS after another researcher[131] reported clusters of cases linked to the same sexual contacts. Klovdahl's work showed direct graphical linkages of the first identified case to eight other patients a cluster of cases identified by the United States' Centers for Disease Control. His approach helped uncover transmission pathways in a disease of unknown etiology and changed the landscape of infectious disease research to incorporate the tools of network analysis.

Analytical and mathematical models of infectious disease often do not adequately capture the important heterogeneous mixing that occurs in real populations.[132] Infectious disease is often transmitted between those who are most closely associated with each other, rather than to random individuals. For example, influenza is more likely to be transmitted among close friends on college campuses[133] and sexually transmitted infections are more likely to be transmitted between preferred sexual partners.[134] Individuals in an outbreak are also heterogeneous in regards to other characteristics, such as individual infectivity.[135] Network analyses explicitly capture and characterize heterogeneity and interdependence among individuals in the spread of a disease. With the increasing global connectedness of individuals and the variety of relationships at different scales, these models become even more critical for both understanding disease and

explaining potential control measures. Fortunately, the wide application of network analyses across numerous disciplines (e.g., computer networks, electronic communication, social sciences, marketing, psychology, education), has led to the tremendous growth in methodologies, which are matched by advances in computational capabilities.[136]

The term 'network analysis' can be used to refer to a variety of different methods and analytic approaches. They are tied to the umbrella term 'network analysis' by the incorporation, at least conceptually, of weighted or unweighted matrices that describe relationships between the different entities (i.e., people, animals, etc.) being studied.[137] Most analyses fall into one or more of three broad categories:[137] 1) network visualization using graphical techniques; 2) descriptive analyses of network topology; 3) modeling diffusion of a process to test inferential hypotheses using statistical modeling or computer-based simulation. Analytic approaches are challenged by the non-independent nature of network data and, therefore, require the use of methods and models that can appropriately deal with observation inter-dependence. Each of these major types of analytic approaches provides valuable insights when applied in epidemiologic studies.

NETWORK CONSTRUCTION AND VISUALIZATION

One of the key conceptual features of network analysis is depiction of the data and their relationships as a network graph. Networks are composed of "nodes" (or vertices) connected by "edges" (or links).[138] Graph theory provides tools and techniques for evaluating the graphs, such as matrices and matrix algebra. Algorithms developed by computer scientists can then be used to quantitatively describe as well as display the network of linked nodes. A network graph and associated descriptive statistics can be easily implemented with open source software, such as in R (package: igraph[139]) or Pajek.[140] Both are widely used within the scientific community. In all instances, visualizations should be used to help illustrate a particular point, for example, clustering of characteristics within a network (e.g., Christakis and Fowler[141] and Fowler and

Christakis[142]). When networks are large, only visualization of subnetworks or certain elements should be used to ensure visual informativity.[143]

The approach to constructing and evaluating a disease transmission network is dependent on the research question, host and pathogen of interest, desired analytic approach and available data. All of these pieces should be taken into account when defining the network nodes and the edges between them. The vast and continually growing literature illustrates the numerous ways to define nodes and edges. Nodes are often defined as individual people and edges represent a defined relationship between the nodes. For example, edges can represent direct sexual contacts[130,144] places of social gathering,[115,145] and family, friends, co-workers, and neighbors.[133,141,142] Spatial proximity and overlap are commonly used to define edges among individuals or groups of modeled wildlife populations.[146–148] In the veterinary literature, nodes often represent locations, such as slaughter houses, markets or farms whereby the relationships between them reflect animal movement.[149–151] Pathogen networks have been constructed to enhance understanding of infectious disease epidemiology. In these networks, edges have been defined as social connections,[78] pathogen sharing,[152–154] and ancestry determined by a molecular clock.[117] Recent reviews provide some guidelines for assembling disease networks for wildlife[155,156] and integrating molecular epidemiology and social network analysis.[157]

Creating a visual network graph is a simple and powerful method that can greatly aid in understanding the structure of the system. Klovdahl[130] used network visualization alone to help uncover transmission structure of an unknown disease. Cook *et al*.[145] improved epidemiologic contact investigations by applying social network analysis to TB outbreaks in California, Georgia, and Vancouver. Many of the patients were not previously linked through conventional contact-investigation data, but clustered together in a social network when linked through mutual contacts or places of social aggregation. McElroy et al.[158] and McKenzie et al.[159] used network

visualization to identify individuals central to ongoing outbreaks. All of these studies discussed network visualization as an aid decision-making by prioritizing resources during disease investigations.

NETWORK TOPOLOGY

The topology of a network is the arrangement of all its elements, including the nodes, edges, components, and their determinants. Studies examining network topology include descriptive analyses of the positioning of nodes, the structural properties of the network, and the patterns of connections across the network.

Individual positioning

The position of particular nodes in a network can be used to describe whether individuals are connected in a way that explains observed patterns. In epidemiology, the position of a node relative to other nodes can have consequences on how a disease will spread. For example, an infected individual on the periphery of the network may not have as much opportunity to transmit disease to others as an individual in the central part of the network.[160] Several individual-level network measures have been adapted mathematically to capture subtle, but important, variations in research questions and data types. A few important concepts are described below, however more extensive descriptions of individual-level network measures can be found in text books and reviews.[137,138,160,161] Centrality is a measure of importance at the individual-level, i.e., the extent to which a node occupies an important (central versus peripheral) location in the network. Network analysts have developed numerous ways to measure centrality, but the most common are degree, eigenvector centrality, betweeness, and closeness.[162,163] Degree centrality, in its simplest form, is the sum of the number of edges connected to other nodes. A higher degree centrality means an individual is connected to more individuals, and therefore is a more important node in the network. Eigenvector centrality is a more complex form of degree centrality that takes into

account whether neighboring nodes are linked to highly connected other nodes. Betweeness centrality is a measure of the extent to which a node occupies a strategic point in a network, serving as a bridge or conduit between groups of nodes. Closeness centrality measures the mean geodesic distance from one node to all other nodes on the network. For disease spread, a node with a short geodesic distance to others (i.e., can easily reach all other nodes) could be a greater concern for quick dissemination of infection.[136] Depending on the research question, one or more centrality measures may be useful for understanding high-risk groups or likely transmission paths.

Contact heterogeneity as measured by centrality can be an important driver of disease. In some instances, it has led to the "superspreader" phenomenon where a few individuals who are highly connected disproportionally drive the spread and maintenance of disease in a population (reviewed by Stein[164]). Superspreading is a characteristic of disease transmission that occurs to a greater or lesser extent in several human diseases, including SARS, measles, HIV, smallpox, and leishmania.[135] A study by Rosenberg *et al*.[165] showed that a high syphilis transmission rate in Louisiana, USA could be maintained by a few individuals that were centrally positioned in the network. Gardy *et al*.[78] used genetic data to confirm that a few individuals identified with high centrality in the social network likely acted as superspreaders, passing *M. tuberculosis* to several other patients. In wildlife, brushtail possums (*Trichosurus vulpecula)* and deer mice (*Peromyscus maniculatus*) have been shown to exhibit contact heterogeneity, leading to superspreading in transmission of bovine tuberculosis and Sin Nombre virus, respectively.[166–168]

Network structure

Network-level measures provide information about the structure of the entire network, which can also be important in disease transmission. Additional ways to characterize network structure include specific measures of the network. The most fundamental measures of network

topology are size (number of nodes) and density (proportion of observed edges out of total possible).[138] Both of these measures can affect disease spread and have a reciprocal relation. For example, the rate of disease spread may be slower in a larger network, which tends to be less dense. A smaller network that is more dense will tend to have more connections and disease will spread faster.[136] Two network features that can influence the size of an epidemic and speed of disease spread are degree distribution (distribution of the number of connections for each node) and clustering (the extent which nodes create tightly knit subgroups). Measures of the observed network can then be compared to properties that characterize theoretical transmission networks. For example, small-world networks are characterized by mostly local clustering of highly connected individuals, but a few long-range links allow for disease to reach all parts of the network quickly.[169] Scale-free networks show low connectivity for most individuals, but also have a few highly connected individuals that allow disease to spread even when the probability of transmission is low.[169] Numerous additional network measures have been developed to describe how structure influences observed patterns and are elaborated on in several reviews and textbooks.[136,138,161]

Identifying patterns with ERGMs

More complex effects of network structure can be examined using analysis of exponential random graph models (ERGM). A large number of network models are randomly generated based on the observed network that preserve important structural features (e.g., size, density, disease prevalence), but randomly vary the feature of interest to generate a normal distribution of the feature. Calculated statistics from the observed network can then be compared to the distribution of the randomly generated networks to determine if the observation departs from what would be expected by chance.[136] Simulation as described above has been used extensively by Christakis and Fowler to examine social contagion.[170] They determined whether patterns of obesity,[141]

happiness,[142] smoking cessation,[171] and several other characteristics (reviewed in their 2013 paper[170]) were more clustered in an observed network than would be expected by chance alone.

Three major types of analyses are generally used with ERGMs:[136] 1) Characteristics of the observed network (e.g., density, clustering) may be compared to see if they differ from measures on the randomly generated ERGMs. For example, disease spreads more efficiently in highly transitive networks, where the same node can be reached through more than one transmission route.[161,172,173] Determining whether a network has significant transitivity can provide insight on the size and speed of disease spread.[172,173] 2) More specific hypotheses of transmission may be tested by determining whether there is an association between node characteristics and network links. An example of this would be evaluating contagion effects across degrees of separation as described by Christakis and Fowler[170]; and 3) More traditional epidemiologic analyses may also be adapted to include network structure. A multivariable model may evaluate the association between a predictor and an outcome while controlling for node and network properties. For example, Valente et. al.[160] showed that overweight adolescents were more likely to have overweight peers using a random-effects logistic regression, that controlled for network structural effects. Associations between friendship ties and weight were then evaluated by comparing the estimates on the observed network to those generated from ERGMS, to further account for network interdependencies. In each of the three structural analysis listed above, statistical significance can be determined by comparing the generated statistic (e.g., density, clustering coefficient, relative risk, beta coefficient from a logistic regression) to a distribution of that outcome in the ERGMs.

INFERENTIAL MODELING

Analytical and dynamic models provide powerful tools to test inferential hypotheses and study the spread of disease or other phenomena across network ties. While methods in this area

are still evolving to deal with the complex interdependencies in networks, two major approaches have been used: 1) statistical analysis using longitudinal regression; and 2) stochastic agent-based models that simulate dynamic disease spread across a defined network structure.

Longitudinal regression models

Longitudinal regression models have been used to determine whether the spread of phenomena over a network is associated with particular exposures or characteristics of individuals.[160] Event histories of exposure to other nodes on the network are constructed for each subject over time, and then a time-specific network exposure term is incorporated as a predictor in the regression. This estimates the effect of each individual's place in the network on the outcome of interest, while controlling for other covariates, confounders, and network effects. To implement this model, network connections and individual node characteristics must be measured at multiple time points and the time that the outcome occurred must be known. Even with these data, one may not be able to separate effects of contagion (i.e., spread between connected individuals) from homophily (i.e., connected individuals tend to be more alike)[174] from confounding (i.e., concurrent exposures to other factors lead to a similar outcome in connected individuals).[175]

Models incorporate the Markov chain assumption (that the current state only depends on the state at the previous time) are recommended for longitudinal data analysis of networks to control for autocorrelation across observations.[176] Regression models preserving the Markov assumption have been used to both detect[141,142] and not detect[177,178] contagion effects across dynamic social networks. Such models are particularly useful for evaluating dynamic diffusion processes occurring in large networks.

In the absence of longitudinal data, regression models are still useful if they can account for network clustering,[179] or use random permutations or bootstrapping methods (i.e., estimating

the variance of parameters from large numbers of random sub-samples from the network). These methods adjust standard errors and p-values to correctly account for the non-independence of observations.[180]

Agent-based simulation

'Agent-based' or 'individual-based' simulation modeling is a technique that applies a set of rules to computer simulated "agents" (i.e., a person, animal, or whatever entity is being studied) to dictate agent behavior. Varying the rules through simulation creates parameter ranges to understand how the system behaves under various hypothetical conditions.[181] Agent-based models are becoming more common for studying disease spread due to both advancements in computational capabilities and the need to capture individual heterogeneity that drives disease spread. More traditional, mathematical, systems dynamics models assume each individual has the same probability to contact any other individual in the population.[73] That is, the basic model assumes random mixing. Agent-based modeling can incorporate heterogeneity of personal contact networks and incorporate patterns.[182] Thus, disease spread through a network can be simulated using a stochastic individual-based model that captures contact heterogeneity and other individualized traits.

Recognition of the importance of incorporating community structure and heterogeneity of contact into models of dynamic disease spread is increasing. During the 2002 global outbreak of severe acute respiratory syndrome (SARS), variation in infectiousness was observed over several superspreading events, such as that described by Chen et al.[183] and Shen et al.[184] This highlighted the need to use approaches that captured heterogeneous mixing.[132,185] Heterogeneity in contact that subsequently drives disease emergence is found in varying degrees in a number of human diseases,[135] animal populations,[167,168] and other systems, such as the livestock trade,[186,187] and live bird markets.[188]

Nesting the agents within a defined social structure shows great promise to improve understanding of disease emergence populations. First, an observed or theoretical network is created that defines the contact structure. Then, an agent-based model is constructed within the social network framework, which restricts the mixing of some individuals and allows others to play a more central role. The advantage to constructing the model with a network is that it moves epidemiology away from a reductionist approach that centers on identifying "risk-factors" and ignores the interrelatedness of observations; with an agent-based model and a social network, the investigator can examine mechanistic interactions, feedback loops, and reciprocity of social interactions on disease spread.[189] Additional advantages to coupling a social network with agent-based modeling include improved understanding and tests for causal inference as well as forecasting the outcome of policy interventions.[189] For example, a recent study used an agent-based model and a defined network to identify interventions to reduce spread of highly pathogenic avian influenza, subtype H5N1, through live bird markets in Southeast Asia.[150] Another researcher used it to design targeted vaccination approaches to prevent massive outbreaks of a highly contagious disease such as smallpox.[190]

Development of best practices within the agent based methods using social networks are still needed.[189] When research questions align with an agent-based modeling approach, either a theoretical or an observed network can be used. The advantage of using a theoretical network is that social network data about individual-level relationships are not needed. When data on individual relationships are available, then constructing the network and evaluating it with the analytic approaches described in this chapter is a first step towards building an agent-based model.

SUMMARY OF PART 2

In conclusion, network analysis is a powerful analytic tool that arose in other disciplines, but is becoming increasingly essential for studying the epidemiology of infectious diseases. The methods allow researchers to not only describe predictors of disease, but also understand the underlying structure of relationships in a system that gives rise to those predictors and patterns of disease spread. Analytic methods range from visualizations using network graphs, to evaluations of topology that describe node and network level characteristics, to methods for using statistical and agent-based modeling to test inferential hypotheses.

CONCLUSION OF INTRODUCTION

Avian mycobacteriosis is a well-recognized disease of birds, but transmission is not well-understood. Social network analysis provides new epidemiologic tools that can evaluate the underlying transmission structure between individuals, which differs from more conventional epidemiologic methods that focus on the course of an outbreak and the attributes of individuals. Applying social network analysis to elucidate transmission patterns of avian mycobacteriosis promises new insight on the epidemiology of a chronic disease with limited transmissibility.

Bird populations at SDZG offer a unique opportunity to apply a social network analysis to further understand transmission dynamics of avian mycobacteriosis. The historic cohort of SDZG birds is a dynamic population of greater than 16,000 birds (1992 – June 2014) with comprehensive post-mortem disease surveillance and detailed management records tracking complete enclosure and movement histories. Unlike most zoo bird populations, birds housed at SDZG are frequently moved between enclosures for various management reasons (e.g., breeding, aggression, and exhibition), making each bird's exposure to other birds and environmental sources unique over time. This creates a complex web of contact patterns that could facilitate,

impede, or mask disease transmission. It also creates an ideal system to study contagion of avian mycobacteriosis in a completely enumerated population with near-complete data over time.

The goal of this dissertation is to use social network analysis to better understand global transmission patterns of avian mycobacteriosis. The research described herein could have a revolutionizing influence within managed bird populations and conservation programs: it will contribute to a better understanding of disease epidemiology, improve population disease management, and demonstrate the utility of social network analysis to study disease transmission in zoos and managed wildlife populations.

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CHAPTER 2: SPATIOTEMPORAL NETWORK STRUCTURE REVEALS CONTAGIOUS DISEASE PROCESS

ABSTRACT

Objective: Avian mycobacteriosis has long been considered contagious, passing between birds via the fecal-oral route. However, independent environmental sources could also be important. Both transmission pathways are biologically plausible, but differentiating between them in observational research is nearly impossible due to complete confounding of exposure pathways. The objective of this study was to use social network analysis to separate confounding due to common exposure and test for contagion by exploiting spatial and temporal variation of ties within the network structure.

Subjects: The dynamic source population included 16,430 birds housed at San Diego Zoo Global from 1992-2014. Embedded within this population was 13,409 "egos", or study subjects, that were further evaluated.

Methods: Network edges were assembled from enclosure histories and temporally aligned to correspond with probable incubation and shedding periods of individually connected birds. Cases (n=275) were identified when acid-fast bacilli were observed in tissues by histopathology. Relative risk of mycobacteriosis was estimated for direct and indirect contacts with infected birds. Further stratification of ties by spatial and temporal characteristics evaluated contagion and homophily. Significance was determined by comparing observed estimates to those generated with 1000 random network permutations.

Results: Disease clustered significantly among both directly and indirectly connected birds. Importantly, there was a 1.31 factor increase (p=0.004) in disease risk from infected birds (versus non-infected birds) that never resided in the same enclosure and were only connected indirectly through another bird. This provides strong evidence that a contagious process is present, because the association persisted with indirect contacts when confounding due to common environmental exposure was removed.

Conclusions: Associations within the network suggest a contagious process drives some of the observed disease incidence. Analysis of network substructures can serve as a powerful, effective tool for separation of contagion, confounding, and other drivers of disease emergence.

INTRODUCTION

Avian mycobacteriosis has long been considered contagious, passing indirectly between birds through the fecal-oral route.[1,14] However, recent long-term studies in well-characterized cohorts of birds have found low probabilities of disease acquisition among exposed birds.[9,75] Additionally, limited genetic and speciation data from managed populations show multiple strains and species of mycobacteria associated with a single outbreak.[13,81–83] This supports pre-existing environmental reservoirs of potentially pathogenic mycobacteria as a cause of many avian infections, as is the case with non-tuberculous mycobacterial infections (NTM) in humans and other animals.[31,122,127] Both transmission pathways are biologically plausible, but our ability to differentiate between the two in real world populations is nearly impossible: exposure to environments where mycobacteria could have been indirectly transmitted from another bird is the same as exposure to environments where the infection could have been acquired from an environmental source.

Social network analysis can help resolve disease transmission questions by incorporating the structure of relationships between individuals.[137] Spread of phenomena can be explored through tests of clustering of disease among connected individuals within the network structure. Such tests have been extensively applied by Christakis and Fowler[170] and several of their coauthors to evaluate social contagion and disease contagion in networks of people (e.g., Christakis and Fowler,[133,141,171] Fowler and Christakis,[142] and Coviello et al.[191]). Their studies have found that most phenomena attributed to contagious processes tended to show clustering extending up to 3^{o} of separation on a network.[170] Mycobacteriosis resulting

from a contagious process should exhibit patterns of clustering that extend through the network beyond directly connected individuals. Infections acquired from an exclusively environmental source of mycobacteria may also exhibit spatial and temporal clustering, if the environmental sources of contamination are present and exposing multiple birds. This would be analogous to clusters of cases of non-tuberculous mycobacterial (NTM) infections in humans arising from common exposure to environments that harbor mycobacteria, such as soil[115] or heating and cooling devices in hospitals.[114,192] However, an environmental source should not be able to influence patterns of disease beyond directly connected birds when the environmental exposure is different and homophily has been considered.

In the present study, a social network analysis was used to disentangle confounding of transmission routes for avian mycobacteriosis and test for presence of a contagious process. We applied methods to test for clustering of disease by degrees of separation, type and directionality of ties.[170] We further extend these methods by evaluating specific spatial and temporal pathways of connectivity to isolate disease risk attributed to contagion, confounding, and other drivers of disease emergence. This allowed us to test whether a contagious process was present in a dynamic network of birds, and highlighted the value of network substructures to inform disease processes.

METHODS

Source and study population

San Diego Zoo Global houses one of the largest, breeding, zoo bird populations in the world, historically averaging over 3,000 birds at any given time across two facilities, the San Diego Zoo and San Diego Zoo Safari Park (collectively referred to as San Diego Zoo Global, SDZG). Birds are frequently moved among enclosures for breeding, behavior or other management reasons, as well as imported from or exported to other institutions. This creates

a dynamic network of contacts over time that varies individual exposure to environments and other birds.

The source population included 16,837 birds present at SDZG between 1 January 1992 – 1 June 2014 that were at least 6 months old and present for at least 7 days. All birds in this population were under close keeper observation and veterinary care during the entire study period and received complete post-mortem exams if they died. Birds in this population had documented dates of hatch, acquisition, removal, and death. A small number of birds (437 birds) were excluded because they had incomplete information on movements, so that the 16,430 remaining birds had near-complete enclosure tracking over time with move-in and move-out dates for each occupied enclosure. All management data were stored in an electronic database. Thus, the study period targets a population for which 1) avian mycobacteriosis disease status could be determined for study birds if they died; and 2) a near-complete social network of birds could be assembled from electronic housing records.

Identifying cases of mycobacteriosis

If a bird in the source population died, a board-certified veterinary pathologist conducted a thorough post-mortem exam that included histopathology on complete sets of tissues, unless advanced autolysis precluded histopathologic evaluation. If lesions suggestive of avian mycobacteriosis were observed during gross examination or upon review of histopathology, which included routine hematoxylin and eosin staining of tissues, then Ziehl-Neelsen or Fite-Faraco special stains were used to confirm presence of acid-fast bacilli. Occasionally, clinical presentation of disease permitted antemortem diagnosis of avian mycobacteriosis based on tissue biopsy. For the purposes of this study, any bird with acid-fast bacilli present in tissues was considered positive for avian mycobacteriosis at the date of diagnosis.

Birds were classified as 'infected' on their date of diagnosis or 'uninfected' on their date of death if the post-mortem examination showed no evidence of disease. Birds were also classified as 'uninfected' on their date of export if they were still apparently healthy. Birds that were still alive on the study end date of 6/1/2014, were followed for up to the assumed minimum incubation period (e.g., 6 months or through 11/28/2014) to determine final disease status.

Definition of network nodes and edges

The network was defined based on the subset of birds that qualified as egos, their alters, and the connections between them (network edges). The term "ego" is commonly used in social network analysis to describe the study subjects,[161] or in our case, the birds which we assessed for risk of infection. Egos included all birds from the source population with complete information on history of exposure to other birds. This included both birds that hatched in the population, as well as birds imported from elsewhere. If a bird was imported, then it must have been present for a duration equal to or greater than the maximum incubation period (further defined below); those that were present less than the maximum incubation period were excluded as study subjects because they could have been infected prior to importation.

Any bird (including other egos) that directly shared an enclosure with an ego for at least 7 days was considered an "alter". Spatial connections between egos and alters were determined through cross-referencing enclosure move-in and move-out dates of egos with every other bird in the source population. Contact occurring in a few enclosures, including hospital and quarantine enclosures, could not be determined and were therefore excluded. Egos were considered exposed to alters for the duration that the alter was spatially and temporally linked to the ego.

Exposures of egos to alters that could lead to potential transmission of mycobacteriosis would be those which occurred within the incubation period before diagnosis of the disease in the ego. However, the distribution of the true incubation period for avian mycobacteriosis is unknown. As a starting point, minimum incubation period, i.e., the minimum time for an exposure to result in detectable disease, was set to 6 months. This was based on early literature from experimental studies that mimicked natural transmission.[62,92] This is also consistent with our own data where the earliest case in the population occurred at 182 days of age.[9] Maximum incubation period was set to 2 years. Early studies reported deaths occurring up to 12-14 months after infection.[62,92,93] However, some authors reviewed by Feldman[14] considered it possible that the disease progression could take years. For egos that were classified as non-infected at death or at the time of censoring (export or the end of the study), this same interval (2 years to 6 months previous) was used to identify contact with alters. For example, if an ego died on January 1, 2005, it would be connected to all alters with which it shared an enclosure for at least 7 days within the time window of 2 years until 6 months prior to the ego's death, or between 1/12/2003 and 7/5/2004.

Exposures of egos to alters that could lead to potential transmission of mycobacteriosis would also be those which occurred within the alters' infectious periods when the infection could spread to other birds. The period of shedding during which a bird is infectious for other birds is unknown; no estimates were available for duration of infectivity in a naturally occurring disease course. Therefore, alters were assumed to be infectious for the maximum incubation time, or two years, as a starting point (illustrated in Figure 2.1). Exposure of the ego to alters that were not infected was considered for the same two-year period prior to the alter's final date in the study. Figures 2.2a and 2.2b illustrate network assembly over time for an example ego and it's alters.

Social Network Disease Transmission Analysis

The bird network was graphed using the Kamada-Kawai[193] algorithm and all visualizations and analyses were performed using R software (package: igraph[139]). Birds were represented as "nodes" on the network and connectivity was represented by "edges" linking the nodes. An initial network was structured to include all connections of 7 days or more between birds that occurred during their lifetimes. From this, the network used in the analyses was constructed by refining connectivity based on the egos' incubation periods and alters' infectious periods as described above. Network topology was characterized by size (number of nodes and edges), average path length, and transitivity (probability that two connected birds both share a connection with another bird).

Patterns of disease transmission on the network were assessed by estimating the ratio of the probability of disease in an ego given exposure to an additional infected alter relative to the probability of disease in an ego not exposed to an additional infected alter, i.e., the relative risk (RR). To determine significance of the RR, the observed RR was compared to the distribution of the same RR calculation on 1000 randomly generated null networks where the network topology and disease prevalence were preserved, but the disease status was randomly shuffled to different nodes.[170,194] Disease status was shuffled separately among birds that were egos and those that only served as alters to retain the respective prevalence in those separate groups. If the observed RR fell outside the range of permuted values between the 2.5th and 97.5th percentiles, i.e., the null 95% confidence interval (CI), then we rejected the null hypothesis that the observed relationship was due to chance alone. Reported p-values were estimated from the null 95% CI.

Risk of disease transmission was evaluated for 5 types of shared relationships between egos and alters (Figure 2.2b). Each evaluation targeted different groups of ego-alter

pairs that varied in degrees of separation as well as spatial and temporal characteristics of edges.

Relationship 1: Risk of disease transmission associated with 1° contacts. This analysis examined all pairs of birds where the ego was in direct contact with the alter during the defined incubation period of the ego. The RR estimate includes the combined risk from direct exposure to both other infected birds and a common environmental source.

Relationship 2: Risk of disease transmission associated with 2° contacts. This analysis examined whether disease risk increased beyond directly connected birds. To identify 2° alters, a matrix of shortest paths was constructed between all ego-alter pairs that never directly shared an enclosure, but were indirectly connected through an intermediary bird. Before estimating the RR and conducting the random permutation tests, the 2° contacts were further filtered to include only pairs where the alter was "temporally antecedent" to the ego, i.e., it shared an enclosure with the intermediary bird before the intermediary bird contacted the ego. This ensured temporal orientation to include only the 2° alters that could have influenced their egos' outcomes. The estimated RR includes the combined risk at 2° of separation from both exposure through an intermediary infected bird and potentially exposure to a common environmental source.

Relationship 3: Risk of disease transmission associated with 2° contacts sharing environment with their ego. This analysis examined associations with the subset of 2° alters from Relationship 2, where both birds were in the same enclosure but not at the same time. This may have occurred when an intermediary bird was present, bridging the different temporal windows between the ego and 2° alter. Associations in this group would reflect a combination of risk at 2° of separation due to common environmental exposure and contagion.

Relationship 4: Contagion. This analysis examined associations with the subset of 2° alters that were both temporally antecedent and never in the same enclosure as their ego. Spatial separation occurred when the intermediary bird was moved, linking two birds in separate enclosures. This group of alters was further refined to ensure that they had no affiliation with the egos' enclosures at any time during the study period. This comparison was key for removing confounding effects of environmental exposure on the contagious process.

Relationship 5: Homophily. This analysis examined associations with the subset of 2° alters that were both temporally subsequent and never in the same enclosure as their ego. Although disease clustering identified through Relationship 4 would be mostly due to contagion, there is a possibility that some of the association could be explained by homophily,[174] i.e., that directly or indirectly connected birds could be more alike than the general bird population in terms of species, behavior, susceptibility, enclosure characteristics, etc. This could make both birds more likely to acquire infection from any source rather than only through contagion. We tested the network for the presence of homophily by estimating risk of disease transmission from 2° alters in a different enclosure that were temporally subsequent to their ego. These alters were similar to those included in Relationship 4, but could not have influenced the infection status of their ego because they had contact with the intermediary bird after the intermediary bird contacted the ego.

Sensitivity analyses

Sensitivity analyses were performed to compare differences in RR estimates while varying model assumptions. Ego incubation time (testing a minimum of 3 months and a maximum of 1, 3, 4 and 5 years) and alter infectious time (2 years, 1 year, and 6 months) were varied for all 5 types of shared relationships. Alters were also limited to those whose exposure to the ego occurred exclusively outside of the 2-year infectious window. We also

refined network edges to contacts between egos and alters that occurred only in small enclosures where enclosure sharing may be a better proxy of true exposure. Finally, we limited analyses to egos and alters that received a post-mortem examination at SDZG.

RESULTS

The 16,430 birds in the source population represented 950 species and subspecies that were housed across 848 enclosures. Mycobacteriosis was diagnosed in 275 (1.7%) of these birds. In total, 13,409 of these birds served as egos for the analysis, representing 810 different species and subspecies of birds, of which 203 (1.5%) developed disease. Egos were housed across 837 different enclosures that varied in size, housing anywhere from 1 to over 200 birds at any given time. Egos were present in the study population for variable amounts of time with the median follow-up being 3.4 years (IQR: 1.4-7 years). On average, egos moved between enclosures 4.4 times (SD: 4.1; range: 0-71), and were housed in 3 separate locations (SD: 2.5; range 1-26). Their average time spent with each alter was a little less than a year (314 days; SD: 201 days).

The initial network, which included all ego-alter connections that occurred for 7 days or longer had 2,492,438 edges. Most birds were incorporated into a single giant component (n nodes=15,404), but several other smaller components (n components=455) were identified and many of these only contained a single bird that was not connected to others. The refined network, where exposures were limited to those that occurred within the egos' incubation periods and the alters' infectious periods included all 16,430 nodes with 905,499 edges. The median number of alters each ego contacted, i.e., degree centrality, was 105 (IQR: 21-303; range: 0-1435). The refined network exhibited small world properties (Watts 1999) with short paths (average path length = 3.8) and many cliques where groups of birds were all connected

to each other (transitivity = 0.63). A portion of the network diagram that includes positive egos and their directly connected alters is shown in Figure 2.3.

At 1° of separation (Relationship 1), the risk of mycobacteriosis given exposure to an infected alter was 7.0 times greater than the risk of mycobacteriosis given exposure to an uninfected alter (Figure 2.4; $p<0.001$). Significant associations persisted at 2° of separation. When all antecedent 2° alters were included (Relationship 2), the RR of disease given exposure to an infected bird, compared to exposure to an uninfected bird was 1.35 ($p<0.001$). When subset to just the antecedent 2° alters that shared the same enclosure (Relationship 3), the RR was 1.47 ($p=0.004$). Importantly, when subset to just the antecedent 2° alters that were never housed in the same enclosure as its ego (Relationship 4), there was a significant 31% increase in risk of infection among egos that were exposed to an infected alter compared to those exposed to an uninfected alter (RR: 1.31, $p=0.004$). Homophily (Relationship 5) was not identified as a contributor to these associations between egos and alters (RR: 0.95; $p=0.586$).

The sensitivity analyses did not yield drastically different findings than the analyses of the main network and the significance of most associations remained (Table 2.1). Generally, as the egos' incubation periods increased, the magnitude of the RRs at 1° and 2° of separation decreased. This same pattern was observed when the network edges were limited to connectivity occurring 2 years prior to the alters' removal dates (i.e., outside of the alters' incubation windows). Patterns of significance were mostly unchanged when the network edges were limited to just animals with post-mortem exams, and just birds housed in small enclosures. Importantly, significant disease clustering in the test for contagion (Relationship 4) persisted in most examined network variations. The exception to this is when the egos' maximum incubation periods or the alters' infectious periods became more narrowly defined.

Homophily (Relationship 5) was detected only when network edges were restricted to exposures outside alters' incubation periods when long time spans were present (RR: 1.10; p=0.014).

DISCUSSION

Using spatiotemporal network analysis, we found evidence that avian mycobacteriosis can spread through bird social networks. Although connected birds may acquire infection from exposure to common environmental sources and may share features that make them more likely to acquire disease through the environment, our data suggest that there is a detectable and statistically significant component of bird-to-bird transmission.

One of the biggest challenges in determining whether a contagious process is present for pathogens such as mycobacteria with fecal-oral spread is the difficulty in distinguishing between different transmission mechanisms that involve the environment. In one scenario, the environment serves as an intermediate collection site for infectious organisms shed by infected birds. In a network, if each bird passes the infection through the environment to one or more other birds, then infection would spread between individuals in chain- or web-like patterns across a network.[195] In the other scenario, the environment serves as the natural reservoir of mycobacteria that give rise to opportunistic infection. Spatial and temporal clustering of independently acquired infections from an environmental source could occur among birds that are housed together and concurrently exposed to environmental sources that favor mycobacterial growth. Homophily,[174] where connected birds tend to be more alike in species, habitat needs, etc. than the general population and, therefore, may share the same disease susceptibility, could occur in both scenarios. For directly connected individuals in our study, the significantly elevated RR represented a combination of these three effects. Examining indirectly connected rather than directly connected birds provided a means to

disassociate exposure to another bird from exposure to that bird's environment. Because there was no evidence of homophily among indirectly connected birds, we could use the network structure to test for the presence of contagion. Among egos who were connected through an intermediary bird to infected birds (2° alters) but did not share an environment with them, the significant increase in risk for mycobacteriosis (Relationship 4) represents just contagion. While this very specific subset of edges allowed for deconfounding of environmental and contagious transmission, it required two consecutive infections among a chain of related birds. This ignored most ego-alter pairs, which shared environments where both processes were possible and completely confounded. While our extensive, long-term set of connections in this network allowed detection of contagion using just this subset, the relative risks likely underestimate the true magnitude of bird-to-bird contagion.

Historically, in experimental infection studies, birds have been shown to be susceptible to the infectious bacilli when directly administered, i.e., introduced intravenously, intramuscularly, intraperitoneally, subcutaneously, orally (e.g., Ashour,[66] Pavlas et al.,[70] Tell et al.,[60] Ledwon et al.[61]). Yet, the relevance of direct inoculation to natural transmission has always been tenuous. Studies have often shown little to no transmission when healthy chickens were placed in contact with either diseased birds or their contaminated environments.[58] Therefore, our study provides new evidence, which supports bird-to-bird transmission in natural settings. Our results also suggest that avian mycobacteriosis is not highly contagious. The small world network structure that we identified for birds in the study population should facilitate rapid disease spread and contribute to epidemic-style outbreaks.[196,197] Most birds did not acquire infection even when directly linked to other positive birds. Over time, we have not seen epidemics and the incidence of disease in this population is low (1%).[9] In his review of early experimental studies, Feldman concluded that

transmissibility was low and that bacteria must be given repeatedly over long periods to ensure infection.[14] The network approach was elucidating in this particular scenario, enabling us to uncover subtle patterns of contagion that are not apparent in disease epidemics.

Environmental mycobacteria are recognized as the cause for NTM infections in humans and other animals.[31,122,127] However, a subset of ego-alter exposures could not be defined within the network to remove effects of contagion from risk for environmental infection. Birds that came into an enclosure after an infected alter left cannot be used to partition the environmental effects because of the potential for long-term (potentially up to 4 years) persistence of pathogenic mycobacteria.[2,63] Genetic data from mycobacterial isolates would be a more definitive method of identifying diverse, environmentally sourced, infection events within a shared environment. Limited genetic and speciation data from managed populations have found multiple strains and species of mycobacteria present within a few apparent outbreaks.[13,81–83] Several different species and genotypes of mycobacteria have been identified in this bird population[12] and we know that some birds could not have passed the infection to each other. Additional studies using genetic data could refine relevant transmission pathways or highlight important environmental sources within the network.

In the present study, we used published literature and our own data to identify plausible, initial infectious and incubation periods for avian mycobacteriosis. However, there is certainly misclassification of exposure because the true extents of these periods are wide and unknown. Generally, mycobacteriosis is considered a chronic disease, with an incubation period that can last for months and possibly years.[1,14] It is also thought that animals can insidiously shed the organisms for long periods of time and those organisms can stay viable in the environment for potentially years.[2,63] In sensitivity analyses to address these issues, our relative risk estimates were generally similar when we varied incubation and shedding

periods. The significance found in the relative risks when limiting network edges to those occurring before alters' 2-year incubation period also suggests that some contagious processes may occur before the 2-year window. The exception to this general pattern in the sensitivity analyses was that evidence for contagion was lost when either ego incubation period or alter infectious period became short (≤ 6 months and ≤ 1 year, respectively). It is likely that the shorter incubation times did not allow sufficient overlap of risk periods to link egos to alters at 2° of separation.

The duration of exposure needed for transmission is unknown, but birds can be housed together for a year or more and not acquire infection.[14,75] Generally, mathematical models show that increasing the intensity or duration of contact between individuals with an infectious disease increases the probability of a transmission event.[195] In the present study, we required a minimum of 7 days together to establish a network link that could capture relevant, short-duration exposure; however, the majority of birds were together for longer, with the mean contact-days being about 10.5 months (314 days). The indirect transmission route of avian mycobacteriosis creates challenges to estimating meaningful contact duration due to environmental persistence of mycobacteria.[2,63] Further exploration of effects of contact heterogeneity on network associations may provide additional insight into clinically relevant exposure duration.

Although we were able to determine a definitive disease status for birds that died, other birds that were true positives could have been misclassified as negative if they left the zoo population or the study ended before they were diagnosed. Birds would not be exported if not apparently healthy and most received pre-shipment physical exams and sometimes diagnostic tests. Such exams are not always sensitive to picking up early stages of infection (reviewed by Tell et al.[7]). To ensure associations were not driven by this potential disease

misclassification, a sensitivity analysis was performed analyzing associations among just birds with post-mortem exams at SDZG. The direction and significance of the RRs were the same as those identified in the main network. If such misclassification errors did exist, they should bias estimates towards the null.[73]

Many of the issues that cause bias in network measures, such as node censoring,[198] or network boundary specification[136] are unlikely to have affected our findings. Our network was created from a closely monitored zoo population with complete records on immigration, emigration, and housing history of all individuals for over 20 years. Coupled with continual population health monitoring and pathology findings on almost every bird that died, these data are unparalleled in terms of the completeness of the network during the observation period. We took care in assembling the network to ensure that the edge construction between egos and alters adhered to general recommendations for disease networks.[155,156,199] This included incorporating biologically meaningful time-periods relevant to mycobacterial disease ecology and the type of exposure needed for transmission. We also used stratified analyses, aligned data temporally and conducted sensitivity analyses on the parameters we did not know to ensure the network was constructed to test the hypotheses of interest. As our network edges became more refined to relevance for disease transmission (i.e., near the alters' study end date), the magnitude of associations increased.

Our approach to isolating confounding of disease transmission pathways using 2^o of separation or 'friends of friends' and partitioning the network structure is new. Inferring contagion by testing for disease clustering in subsets of the network requires quite complete network ascertainment, very good information on location over time, and a large number of egos and alters to create the network edges. This allowed us to use spatial and temporal characteristics of the network to select sets of egos and alters that never shared enclosures and

thus remove confounding of the environment on transmission mode. While we were able to test whether contagion was occurring, we could not specifically adjust for confounding to obtain unbiased estimates of the magnitude of RRs. Our findings show that contagion does play a role in disease acquisition.

Most epidemiologic studies that use a network approach focus on directly transmitted, infectious diseases.[155] Social networks to investigate diseases transmitted indirectly are assembled less often because defining contact in the presence of environmental persistence or other important transmission routes, such as fomites or insects, can be challenging.[156] To our knowledge, this is the first application of using contagion theory and disease clustering on a network to determine whether patterns of connectivity show an infectious versus a non-infectious process. Similar approaches could be useful to investigate diseases of humans or animals when the network is complete but the disease etiology is unknown.

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Table 2.1— Sensitivity analyses of the relative risk of avian mycobacteriosis given exposure to an infected enclosuremate in San Diego Zoo Global's bird population, 1992-2014 (n=16,430 birds).

Sensitivity analysis	Egos	Edges	Relative risk and p-value[a] Relationship 1[b]	Relationship 2[b]	Relationship 3[b]	Relationship 4[b]	Relationship 5[b]
Main network							
ego incubation min 180 days, max 2 yrs; alter infectious 2 yrs	13,409	905,499	7 p<0.001	1.35 p<0.001	1.47 p=0.004	1.31 p=0.004	1.18 p=0.586
Modified ego incubation: minimum ,maximum							
90 days, 2 yrs	13,402	969,958	7.03 p<0.001	1.44 p<0.001	1.38 p<0.001	1.45 p<0.001	1.05 p=0.586
180 days, 1 yr	14,655	693,347	7.24 p<0.001	0.97 p=0.779	1.54 p=0.010	0.77 p=0.071	0.78 p=0.097
180 days, 3 yrs	12,567	1,041,593	4.35 p<0.001	1.35 p<0.001	1.29 p=0.022	1.36 p<0.001	1.08 p=0.263
180 days, 4 yrs	11,924	1,142,294	3.43 p<0.001	1.34 p<0.001	1.62 p<0.001	1.27 p<0.001	1.03 p=0.674
180 days, 5 yrs	11,352	1,227,119	2.99 p<0.001	1.3 p<0.001	1.6 p<0.001	1.23 p<0.001	1.02 p=0.763
Modified alter infectious period							
180 days	13,409	542,181	7.31 p<0.001	1.23 p=0.040	1.47 p=0.013	1.13 p=0.327	0.87 p=0.308
1 yr	13,409	707,014	7.62 p<0.001	1.25 p=0.006	1.45 p=0.003	1.18 p=0.142	0.86 p=0.19
Birds with post- mortem data	5,369	905,499	3.3 p<0.001	1.24 p<0.001	1.46 p<0.001	1.16 p=0.002	0.96 p=0.476
Only connections through small enclosures	11,069	204,847	5.19 p<0.001	1.39 p=0.005	1.47 p=0.065	1.35 p=0.044	1.07 p=0.738
Contact only 2 or more years before the alter's removal	13,409	604,078	1.54 p<0.001	1.12 p<0.001	1.21 p<0.001	1.08 p=0.025	1.10 p=0.014

RR=Relative Risk; CI= confidence interval. Significant associations (p<0.05) are shown with a gray background.

[a]The calculated statistic is the probability that an ego has disease, given that its alter has disease, compared to the probability that an ego has disease given that its alter does not (i.e., RR). To determine whether the observed RR falls within the 2.5th and 97.5th percentile of the null distribution, the disease status was randomly reshuffled in 1000 network permutations where the network structure and prevalence of mycobacteriosis was preserved. Significant p values indicate the observed RR fell outside of the null 95% CI and we reject the hypothesis that the observed RR is due to chance alone.

[b]The five evaluated relationships are described in detail in the Methods and in Figure 2b.

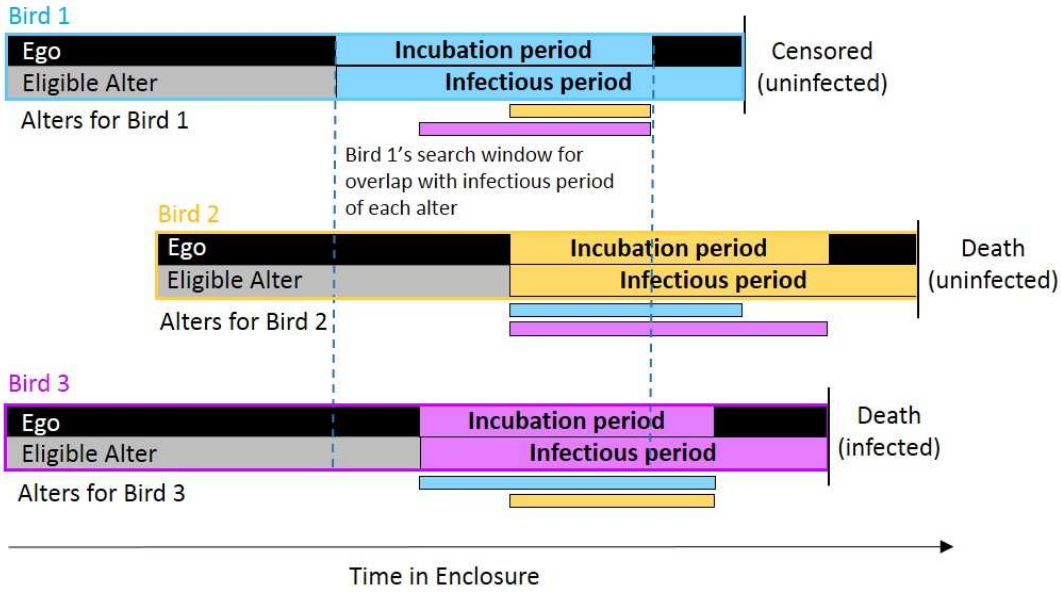

Figure 2.1— Diagram of network edge construction. The figure represents three birds. Each bird can serve as an "ego" (i.e., study bird) and/or an "alter" (i.e., bird connected to the ego), depending on where they lived and the timing of their overlap. Any bird that shared an enclosure with the ego during its "incubation period" could serve as an alter for that ego, provided that the timing of overlap occurred within the alter's "infectious period". Each ego's incubation period was initially set to the period occurring 6 to 24 months prior to the ego's final date in the study. Each alter's infectious period was initially set to the period occurring 2 years prior to its final date in the study.

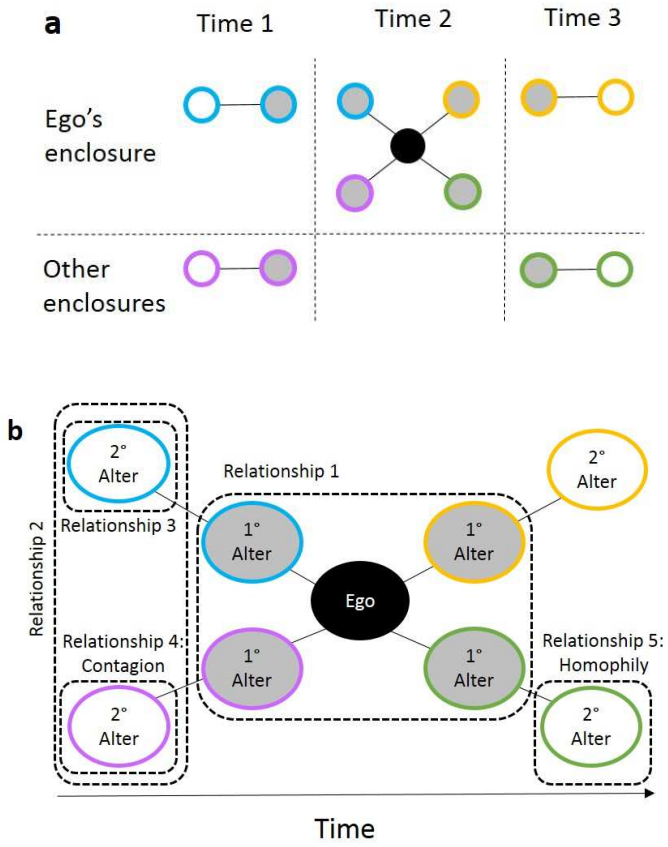

Figure 2.2— Illustration of network assembly and evaluated relationships. Each circle represents a different bird, showing alters at 1° (gray) and 2° (white) of separation related to one ego (black). Colors represent pathways along which the different evaluated relationships were formed. Lines represent the network edges which connect birds that shared enclosures. 2.2a: Illustration of network construction. Second degree alters differed by time (antecedent vs. subsequent) and space (same enclosure vs. other enclosure) with respect to their connection with their ego. 2.2b: The ego's assembled network of 1° and 2° alters. Evaluated relationships using relative risk and random permutation tests: Relationship 1: Risk of disease associated with direct contacts. Relationship 2: Risk of disease associated with indirect contacts that are temporally linked in the past, or "antecedent", to their ego. These are the 2° alters that could have influenced their ego's disease status. Relationship 3: Risk of disease associated with antecendent, indirect contacts that are affiliated with the same enclosure as their ego. Disease risk from these birds represents a mixture of infection acquisition from environmental sources and contagion. Relationship 4: Contagion. Risk of disease associated with antecedent indirect contacts that are affiliated with a different enclosure than their ego. This evaluation is key for removing the confounding effects of the environment and testing for a contagious process. Relationship 5*:* Homophily. Risk of disease due to temporally subsequent, indirect contacts affiliated with a different enclosure than their ego that could not have transmitted disease. This tests for the presence of homophily, i.e., whether egos are more similar their 2° alters than other birds on the network.

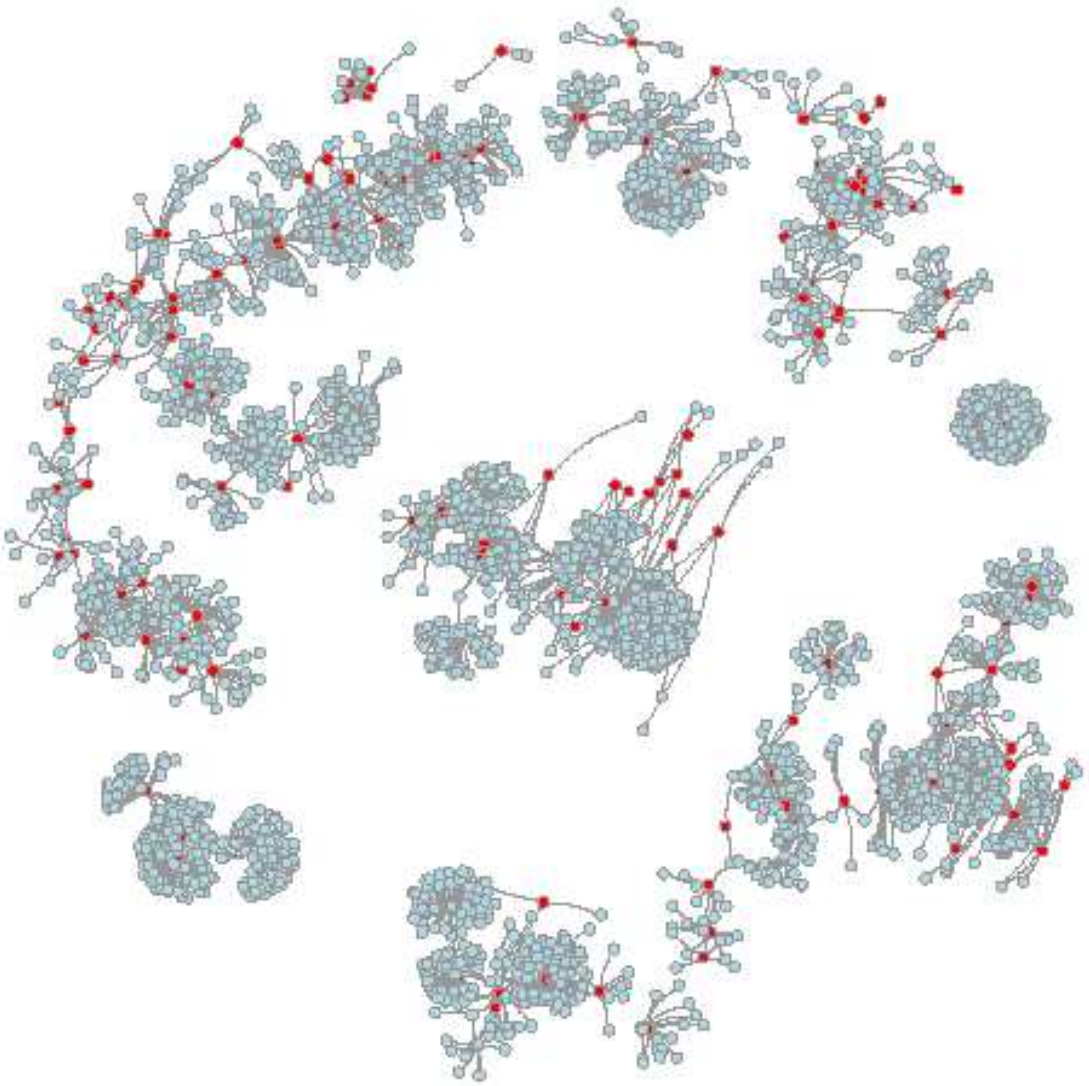

Figure 2.3— Social network graph of a subset the San Diego Zoo Global bird network, 1992-2014. The subset of the network illustrates all positive study birds ("egos") and their direct contacts ("alters"). Each node represents one bird in the data set and connections between birds were defined by enclosure sharing. There are 3417 birds represented in this subset with 6066 unique connections between them. The color of the circle indicates each bird's disease status: red denotes a bird with mycobacteriosis and light blue denotes a bird that did not have disease. Statistical tests for clustering of disease on the network showed statistically significant increases in disease risk for an ego directly and indirectly connected to infected alters, compared to an ego directly or indirectly connected to an uninfected alter.

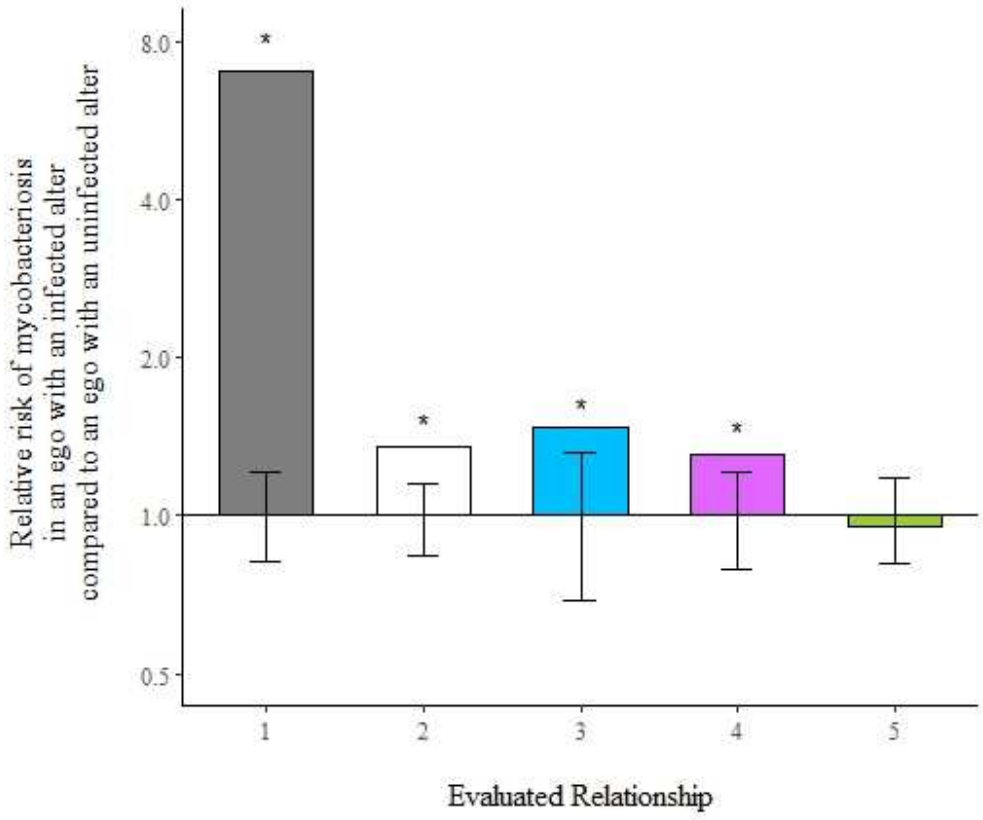

Figure 2.4— Relative risk estimates of the main network. The estimated RR for each of five different relationships (described in the text) between egos and alters that were connected at 1° (Relationship 1) and 2° (Relationships 2-5) of separation is shown. Significance of the estimate was determined by comparing conditional probability of mycobacteriosis in the observed network with 1000 permutations of an identical network (with the topology and incidence of mycobacteriosis preserved) in which the same number of infected birds were randomly distributed. Error bars show the null 95% confidence intervals generated from the random permutations. RRs that were outside of the null and significant are indicated with *.

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CHAPTER 3: SOCIAL NETWORK ANALYSIS AND WHOLE-GENOME SEQUENCING OF AVIAN MYCOBACTERIOSIS IN A LARGE, DYNAMIC POPULATION OF BIRDS

ABSTRACT

Objective: To determine whether whole-genome sequencing (WGS) and a social network analysis reveal general patterns of contagious disease spread in a large population of zoo birds.

Subjects: Cases (n = 275) of avian mycobacteriosis nested in a source population of 16,430 birds at San Diego Zoo Global (SDZG) facilities between 1/1/1992 and 6/1/2014.

Methods: Mycobacteria species were determined using conventional methods and whole genome sequencing and compared across avian taxonomic groups. A social network was constructed from the source population to identify directly and indirectly connected cases during time periods relevant to disease transmission. The WGS data were used to estimate the proportion of connected birds with a similar genotype and determine whether the observed proportion was significantly different than random using network permutations. Among birds with *Mycobacterium avium avium* (MAA) and *M. genavense*, pairwise single nucleotide polymorphisms (SNPs) were determined. Distributions of observed SNPs along paths of network connectivity were compared to random permutations with the Kolmogorov-Smirnov test for equality of distributions.

Results: MAA and *M. genavense* were the most common species identified and they occurred disproportionately across bird taxa. Some genotypes clustered along pathways of bird connectivity, while others were dispersed throughout the network. The proportion of directly connected birds having the same mycobacterial genotype was 0.34 and significant. This proportion was higher (0.54) and significant for MAA, but was not significant for *M. genavense*. Evaluations of SNP distributions showed genotypes of MAA were more related at 1° and 2° of separation than expected by chance; however, no significant patterns of genetic relatedness were identified for *M. genavense*.

Conclusions: Integrating large-scale bacterial WGS and a social-network revealed significant genetic clustering along pathways of connectivity, namely for MAA. Findings are consistent with a contagious process occurring in some, but not all, case clusters.

INTRODUCTION

The epidemiology of avian mycobacteriosis is not well-understood. This chronic disease of birds with an insidious onset and variable incubation is generally considered to be contagious via the fecal-oral route.[1] However, both historic (reviewed by Feldman[14]) and recent studies[9,75] support only low bird-to-bird transmissibility. Other studies[13,81–83] have found diverse mycobacteria from clusters of cases, suggesting that environmental sources[5] could drive disease incidence in birds similar to that in humans and other animals.[31,122,127] When spatial and temporal disease clusters arise, it is unknown whether they result from direct transmission from an infected bird or whether a group of susceptible birds shared exposure to a common environmental source. These two scenarios have different implications for disease prevention and the management of birds in zoos and conservation programs.

Combining social network analysis with whole genome sequencing (WGS) could improve understanding of these transmission pathways. The social network would provide important visualization and capture contact heterogeneity, while genetic data would provide resolution to identify true transmission dynamics.[157] This approach has been used in human studies of *Mycobacterium tuberculosis*[78] and may help elucidate transmission pathways for mycobacterial infections in birds.

In a previous study using WGS to characterize mycobacteria in birds from the San Diego Zoo and Safari Park (collectively referred to as San Diego Zoo Global; SDZG),[12] we found high diversity between individual isolates, but also groups of closely related genotypes. Inferring transmission from WGS data alone was not possible because of incomplete sampling and lack of

information on complex temporal contact patterns between birds. In a second study, we evaluated general patterns of disease spread by examining direct and indirect connectivity of cases, using spatial and temporal variation in the network structure to isolate patterns attributed to contagion (Chapter 2).[170] Cases of mycobacteriosis were significantly clustered in a way that was highly suggestive of a contagious process. However, we could not distinguish between clusters arising from similar versus genetically diverse mycobacteria. The goal of the current study was to provide a more specific test for contagion and improve understanding of disease epidemiology by coupling WGS with a social network analysis. Findings from this study provide additional insight to the complex epidemiology of avian mycobacteriosis.

METHODS

Source population

The source population included 16,867 birds present at SDZG between 1/1/1992 and 6/1/2014. This included all birds that were six months old or older, and living within SDZG facilities for at least 7 days during the study period. Birds in this source population were under continual health monitoring by keepers and veterinary staff throughout the study period and received post-mortem exams if they died. The population was dynamic, with birds being imported, exported, and moved between enclosures for breeding or other management reasons. This housing history was tracked electronically over time and included individual-level information on the specific enclosure and when each bird moved in and out. These enclosure moves captured potential exposure to other birds infected with avian mycobacteriosis. Enclosure-sharing could not be determined from housing history records for 437 of the birds, so these birds were removed from the study. The final population of 16,430 birds represented 950 species and subspecies and was used to identify all birds diagnosed with avian mycobacteriosis and create a social network to link connected cases.

Case identification

Within the source population, 275 birds were diagnosed with avian mycobacteriosis. When a bird from the source population died or was euthanized, a board-certified veterinary pathologist conducted a thorough post-mortem exam that included histopathology on complete sets of tissues unless advanced autolysis precluded evaluation. If gross or histopathologic examination revealed lesions suggestive of mycobacterial disease, then Ziehl-Neelsen or Fite-Faraco special stains were used to confirm the presence of acid-fast-bacilli. Any bird with acid-fast bacilli present in tissues was considered positive for avian mycobacteriosis. Most cases were identified post-mortem, but occasionally clinical presentation permitted diagnosis from a biopsy.

Network construction

Among the 275 cases, 203 birds were identified as the study subjects, referred to as "egos". A case was considered an ego if it had complete exposure history to other birds. This means that the bird either hatched at SDZG or was imported and observed in the population for a presumed maximum incubation time of at least two years. A network was then constructed that linked egos to all other birds in the source population, including those that did not meet criteria for being an ego. These connected birds are referred to as "alters". Each ego could have multiple alters because they were housed with multiple birds during the target periods and many birds served as both egos and alters. Birds from the source population that were not cases were included in the network to define 2^{o} contacts between cases, and then were not included in further analyses.

The network was assembled in a manner similar to that previously described (Chapter 2), defining connectivity between egos and alters as when two birds shared an enclosure for at least 7 days during the ego's incubation period and its alter's infectious period. The ego's incubation periods were assumed to be the time period ranging from 6 to 24 months before the ego's date of

diagnosis. Six months was estimated as the minimum incubation time, which was consistent with early experimental transmission studies in birds[62,92] and with our own observations of the earliest case occurring at 182 days of age.[9] Two years was estimated as the maximum incubation time. Early experimental studies report deaths from avian mycobacteriosis 12-14 months after infection;[62,92,93] however, some experts believe it could take years for a bird to succumb to the disease.[14] No information was available for plausible time periods when an alter may shed mycobacteria. Therefore, the alters' infectious times were set to the maximum incubation time of 24 months prior to the alters' final date in the study, which corresponded to death dates, removal dates, or the end of the study.

Determination of genetic relatedness of mycobacteria

Isolation and species determination of mycobacteria from infected birds were attempted for 167 of the 275 cases of mycobacteriosis. Fresh or frozen tissues (other than feces) were collected using aseptic techniques and submitted to either the Molecular Diagnostics Laboratory (SDZG Institute for Conservation Research, Escondido, CA) or an external microbiology laboratory (University of California San Diego Health System Clinical Laboratory, La Jolla, CA; National Jewish Health Advanced Diagnostic Laboratories, Denver, CO; National Veterinary Services Laboratory, Ames, IA; or University of Wisconsin, School of Veterinary Medicine Mycobacteriology Laboratory, Madison, WI) for mycobacterial culture and species determination using DNA probes, HPLC, or Sanger sequencing.

DNA was extracted from isolates that were viable at the time of the study using QiaAMP DNA Mini Kit (Qiagen, Valencia, CA) following the manufacturers protocol with the pretreatment steps previously described.[12] When at least 0.3 μg of DNA could be extracted, the sample was sent to The Scripps Research Institute Next Generation Sequencing Core (La Jolla, CA) for WGS on a HiSeq 2000 or a NextSeq 500 (Illumina, La Jolla, California). Candidate

SNPs for each isolate compared to a reference genome of the same species were computed using the GATK 3.5 HaplotypeCaller tool (https://software.broadinstitute.org/gatk). Custom scripts were then used to retain only the high-confidence SNPs and to compute the genomic distance in number of SNPs between each pair of isolates of the same mycobacterial species. Detailed sequencing protocols, computational bioinformatics workflows, and all characterized sequences of mycobacteria have been previously reported (uploaded to the NCBI Sequence Read Archive under Bioproject PRJNA351843).[12]

Similarity in genotypes of connected ego-alter pairs at 1° and 2° of separation were assessed in two separate ways. Dichotomously, genotypes were classified as "similar" (i.e., likely part of the same transmission chain) if they were within 12 SNPs of at least one other genotype in a phylogenetic group generated using RAxML 8.2.9 (https://github.com/stamatak/standard-RAxML) as previously described in detail. The threshold value of 12 SNPs was used as an indication of the maximum possible genetic diversity within and between hosts as previously defined for *Mycobacterium tuberculosis*.[77,200] If WGS was not available for both of the birds, then the edge was classified as "unknown". The second method was restricted to comparing MAA and *M. genavense* sequences, separately. A custom Perl script was used to determine the minimum SNP between pairs of birds and this was used as a measure of the relatedness of the cases.

Statistical and network analyses

Mycobacterial species identified from the 275 cases were summarized by bird taxa and isolation method. Frequency of isolation of MAA and *M. genavense* were compared between taxonomic orders where >10 birds had mycobacterial species data. Proportions of birds with MAA versus *M. genavense* were compared with a Fisher's exact p-value.

Network visualizations and analyses were performed in R software (package: igraph[139]). The network of 275 cases was graphed using the Fruchterman-Reingold algorithm[201] to illustrate

connectivity between cases at 1° and 2° of separation and show the four most prevalent genotype groups. Two different node centrality measures were used to characterize importance of case node to the overall network of 16,430 birds: degree centrality (the number of connected nodes) and eigenvector centrality (the extent to which a node is connected to other highly connected nodes).[161] Degree and eigenvector centrality distributions were compared between birds with MAA and *M. genavense* as well as between those with known and unknown genotypes according to Kolmogorov-Smirnov test for equality of distributions.

We determined the proportion of egos and alters having similar genotypes as the number of ego-alter pairs with similar genotypes divided by the total number of connected pairs with known genotypes for both birds. This proportion was then compared to the distribution of the same calculation on 1,000 randomly generated null networks where the network topology and prevalence of each genotype was preserved, but the genotypes (including unknown genotypes) were randomly shuffled to different nodes using methods previously described.[170,194] If the observed proportion was outside the range of the 2.5th and 97.5th percentiles of the null distribution (i.e., the null 95% confidence interval), then the null hypothesis that the observed proportion could have arisen from chance was rejected. This calculation was performed for all directly and indirectly connected birds at 1° and 2° of separation, and among just those with MAA and *M. genavense*, separately. Of note, at 2° of separation, ego-alter pairs were limited to those where the alter was temporally antecedent and housed in a different enclosure than its ego. This important subset of 2° alters provided appropriate comparisons for isolating patterns of contagion within the network structure as previously described (Chapter 2).

To determine whether specific genotypes of connected cases were more related than unconnected cases, numbers of pairwise SNPs between pairs of birds with MAA (n=40 birds; 26 pairs) and *M. genavense* (n=39 birds; 20 pairs; 2 birds were excluded because sequence

comparisons were not available) were summarized separately for ego-alter pairs with WGS. The pairwise SNP distribution for directly connected ego-alter pairs was determined. The observed distribution of SNPs was then compared to the distribution generated from 1,000 random permutations as described above. Significance was determined with the Kolmogorov-Smirnov test for equality of distributions. This was repeated using genetic relatedness for ego-alter pairs connected at 2° of separation.

RESULTS

Population summaries

The 275 cases of avian mycobacteriosis represented 149 species of birds. On average, 12 cases were diagnosed per year (SD=5; range 4-20). The median time spent in the population was 4.5 years (1,638 days; interquartile range or IQR: 2 - 8.2 years; range: 33 days - 26 years). Excluding quarantine and hospitalization-related moves, cases moved on average 5 times during the study period (SD=4.5) and were associated, on average, with 3.5 different enclosures (total represented enclosures among cases = 377).

Species of mycobacteria were determined for 124/275 of the infected birds (45%; Table 3.1). MAA was most commonly identified (52/124; 42%), but was also the most frequently tested for (i.e., culture methods were not optimized for *M. genavense* during the early part of the study period). *M. genavense* was identified in 44 birds (out of 124; 35%). Of the birds with WGS, the numbers with MAA and *M. genavense* were similar (n=40 and n=41, respectively). Nine additional species or subspecies of Mycobacterium affecting 22/124 (17%) cases were identified; 11 of these were *M. a. hominissuis*. Isolates from five birds were identified to the *M. avium* complex level and one was identified as a rapid grower.

Mycobacteria were most commonly found in Columbiformes (pigeons and doves), Anseriformes (waterfowl), and Passeriformes (perching birds; Table 3.2). MAA was more

common in Anseriformes than Columbiformes or Passeriformes ($p<0.01$ for both comparisons). *M. genavense* was more common in Passeriformes than both Anseriformes and Columbiformes ($p<0.01$ and $p=0.02$, respectively), and more common in Columbiformes than Anseriformes ($p<0.01$).

For the cases in the present study, 112 distinct sequences from 97 birds (36% of 275) were obtained. This included 15 groups of genetically similar mycobacteria containing 2 or more birds (7 separate groups of *Mycobacterium avium avium* or MAA, representing 25 birds; 7 separate groups of *M. genavense*, representing 35 birds; 1 group of *M. a. hominissuis*, representing 2 birds). Many birds (n=35) had sequences far apart from all other isolates, including seven additional species of mycobacteria as well as distinct isolates of MAA, *M. a. hominissuis*, and *M. genavense.*[12]

Network analysis

The social network with all 275 cases (nodes) and their connections (edges) at 1° and 2° of separation consisted of 461 edges (338 between the eligible ego-alter pairs) that directly connected 157 of the cases to each other, totaling over 77,000 bird-days of direct case-case exposure. An additional 79 birds were linked to other cases by 2° of separation. Thus, 86% (236/275) of all cases were directly or indirectly connected at 1° or 2° of separation. The four most prevalent genotype groupings, along with known and unknown genotypes are shown (Figure 3.1). Temporal and spatial clusters of both similar and dissimilar genotypes were visually observed, and some genotypes were dispersed throughout the network.

No differences in centrality measures in the network were identified between birds with MAA and *M. genavense* (Kolmogorov–Smirnov test $p> 0.05$ for both measures; Table 3.3). There was some evidence that birds with missing genotype data were more central in the network than

those where the genotypes were known (Kolmogorov-Smirnov p=0.20 for degree and p = 0.047 for eigenvector centrality).

Genetic data were available for both directly connected ego-alter pairs in 61/338 (18%) pairs. The proportion of directly connected birds with a similar genotype was 0.34 and significantly different than random (null 95% CI: 0.00 to 0.13). This proportion was higher when limited to the 26 pairs where both the ego and the alter had MAA ($p_{match|connected}$=0.54; null 95% CI: 0.00 to 0.22). The proportion was not significant among the subset of 24 directly connected birds which both had *M. genavense* ($p_{match|connected}$=0.29; null 95% CI: 0.0 to 0.50). None of the 11 birds with *M. a. hominissuis* were connected, and therefore network associations were not evaluated.

Data were sparse for evaluations at 2° of separation. Genotypes were known for 73/516 ego-alter pairs (14%); however, only 12 of these had the correct spatial and temporal alignment for evaluating hypotheses related to contagion. Among these 12 ego-alter pairs, the proportion having a similar genotype was 0.09 (null 95% CI: 0.08 to 0.22) and not significantly different than random permutations. Birds were not evaluated separately within mycobacterial species groups at 2° of separation because there were only 8 MAA pairs (1 pair had ≤ 12; 7 pairs had > 12 SNPS) and 0 *M. genavense* pairs (the other 4 pairs were birds with different species of mycobacteria).

Density plots showed SNP distributions between ego-alter pairs in the observed and randomly generated networks (Figure 3.2). Directly connected birds with MAA (n=26) were significantly more similar (based on SNPs) than expected by chance alone (Kolmogorov–Smirnov test, $p = 6.08 \times 10^{-6}$). This pattern was still significant at 2° of separation, despite having a very small number of ego-alter pairs with MAA (n=8; Kolmogorov–Smirnov test, p = 0.016). For evaluations among ego-alter pairs with *M. genavense* (n=20), there were no significant

differences in SNP distributions between directly connected birds in the observed and random networks (Kolmogorov–Smirnov test, p = 0.17). Data were too sparse to evaluate the association at 2° of separation for birds with *M. genavense*.

DISCUSSION

Mycobacterial species data were available for nearly half of the birds diagnosed with mycobacteriosis over the 22-year study period at SDZG. In this large, and fully enumerated population of diverse birds with post-mortem surveillance, 62.9% (78/124) of characterized Mycobacterium isolates were MAA or *M. genavense*. This finding is consistent with other reports.[9–12,74,81] Therefore, understanding the transmission dynamics of these two species is an important consideration for managing avian population health.

There was greater genotypic similarity in isolates among cases which shared locational and temporal connections. This pattern was present when pooling data across all species of Mycobacterium, and when limited to just birds with MAA. It was significant both when assigning plausible cutoffs for transmission events, and when removing the cutoff assumption to examine genetic relatedness based on SNPs. While clustering of genotypes in directly connected birds would be expected with a contagious process, environmental point sources of infection could also produce genetic clusters. For example, similar WGS genotypes have been noted for *M. chimaera* outbreaks in hospitals resulting from a single environmental point source.[202] Among the small subgroup of case birds connected at 2° of separation that never had contact with each other or each other's enclosure, we found more genetic similarity, based on SNPs between connected birds with MAA than would be expected by chance. Within this group, genetic similarities cannot be explained by mutual contact to the same environment, leaving contagion as the main driver of genetic relatedness. This provides strong evidence that a contagious process is occurring among MAA cases.

For *M. genavense* we did not find evidence of contagion. Using our genotyping method, *M. genavense* genotypes were very similar between connected and unconnected cases throughout the network. It is possible that our limited number of cases combined with low genetic diversity[12] led to low statistical power. This would be especially true if the social network was not optimized to capture specific timing and contact structure for transmission. It could also be that *M. genavense* is not as readily contagious as MAA. Others have suggested it has low pathogenicity due to lack of disease among in-contact birds.[18,29] It is also possible that the environment is source for *M. genavense* avian infections in the same way it is for human infections.[107,203,204] Differences in patterns between MAA and *M. genavense* may also reflect differences in host characteristics or sampling efforts. There was no evidence that birds with *M. genavense* and MAA had different opportunities to spread disease based on their location in the network (i.e., no difference in degree centrality or eigenvector centrality). Additional studies clarifying transmission mechanisms and describing genetic diversity are needed to improve understanding of the epidemiology of *M. genavense* infections.

The measure of genetic similarity assumed that ≤ 12 SNPs was a sensitive and specific cutoff for identifying transmission events. This cutoff has been used as a threshold for ruling out transmission of *M. tuberculosis* between human hosts[77] and is based on low estimated base pair mutations rates of 0.3-0.5 SNPs per year.[77,205,206] There is evidence that MAA has a similarly low *in vitro* mutation rate of 1 SNP per genome per year.[12] Mutation rates have not been measured for *M. genavense*, but could be lower than other species of Mycobacterium based on the small genomic distance between all of our isolates.[12] Thus, it is possible that the ≤ 12 SNP cutoff does not correctly capture transmission dynamics. Improved understanding of how mycobacteria diversity arises may better resolve transmission.

The proportion of connected pairs having the same genotype was 34%. If we assume that the sampled subset of birds is representative of all cases, then inferring that two clustered cases were caused by the same mycobacteria would have been wrong 66% of the time. These results show that even when an exposed bird becomes infected, it may not be the same pathogen. Other studies have also documented case clusters that were eventually attributed to different mycobacteria using molecular methods.[13,81–83] . Findings from the present study emphasize the need for improved avian mycobacteriosis screening and disease management protocols that address the high rate of false transmission observations. Current recommended protocols focus on breaking the bird-to-bird transmission through halted breeding, reduced movement in and out of exhibits, and depopulation.[1,38,40–43] Improved methods that incorporate epidemiologic findings and genetic data into outbreak investigations could reduce the negative impact of the current disease management approach on population breeding, sustainability, and reintroduction efforts.

Misclassification of network edges may explain some discordance between network connectivity and mycobacteria genomic data. Connectivity between egos and alters was based on defining precise time periods when bacteria could accumulate from another shedding bird; however, enclosure sharing is only a proxy for true contact that would lead to disease transmission. Additionally, the definition did not capture potentially long periods of mycobacteria viability[2,63] that may pose risk of transmission after a shedding enclosuremate is removed. This could have misclassified some birds as not being connected, when they had a true epidemiologic link. We used historical reports[14,62,92,93] and our own data[9] to estimate the incubation and infectious periods, but the true distributions of these important periods are unknown. Sensitivity analyses in our previous study (Chapter 2) showed no major differences in patterns of contagion when the risk periods were modified; however, for the specific pair-based genetic analyses, with our small sample size, this discordance would weaken our ability to detect true contagion.

Genetic data were available for about one-third of all cases, which translated into missing data for 78% of directly connected ego-alter pairs, and 86% of the connected pairs at 2° of separation. This limited our ability to fully evaluate genetic relatedness of mycobacteria within the network. We may also have an incomplete inventory of genotypes among birds with isolates, which complicates epidemiologic interpretations. Infection with multiple mycobacteria has been documented in this bird population,[12] in other birds,[82] and in humans.[207–209] Acquiring WGS data for cases was challenging as it required culture of a slow-growing, fastidious organism to obtain high read coverage which may limit the detection of multiple organisms, if present, or those that do not culture well.[12] Despite the limited data, there were enough pairs of cases with WGS to test for network effects for MAA and *M. genavense*; however there were not enough cases with *M. a. hominissuis* to evaluate patterns of genetic similarity (i.e., only one pair had similar genotypes, and no cases were connected). Following this cohort of birds into the future to obtain additional mycobacterial WGS may fill data gaps.

This study is the first to integrate large-scale bacterial WGS with a social network of birds that provides a framework to examine epidemiology of avian mycobacteriosis in a new way. Our data included complete population identification, diagnostic information on all birds that died, and near-complete housing records for recreating exposure histories. Although genetic data were limited, the resolution of WGS with genome-wide comparisons is superior to conventional DNA fingerprinting for revealing true disease transmission dynamics.[206,210] The results showed that some, but not all, spatial and temporal clusters of cases were genetically similar. Significant patterns of genetic relatedness at 1° and 2° of separation strongly suggest a contagious process is occurring in some situations. Others reveal clusters of cases with genetically unrelated infections. Our findings provide new insights into the complex disease epidemiology and suggest that avian mycobacteriosis is not a single, homogeneous disease entity and that drivers of disease may differ

for MAA and *M. genavense*. Genetic information may need to be considered to optimize control strategies.

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Chapter 3, in full, was prepared for submission for publication of the material. Co-authors include Fowler, James; Pfeiffer, Wayne; Hungerford, Laura; Braun, Josephine; Burchell, Jennifer; Papendick, Rebecca; and Rideout, Bruce. The dissertation author was the primary investigator and author of this material.

Table 3.1— Species of Mycobacterium identified in 124 out of 275 infected birds at San Diego Zoo and Safari Park, 1992-2014.

Species of mycobacteria	Number of birds with mycobacterial species determination by: WGS[a]	other method[b]	Total
M. avium avium			
MAA	37	14	51
MAA & *M. xenopi*		1	1
M. avium hominissuis	11		11
M. genavense			
M. genavense	40	3	43
M. genavense & *M. intracellulare*	1		1
Other species			
M. arupense	1		1
M. fortuitum	2		2
M. hassaicum & *M. peregrinum*	1		1
M. intracellulare	2	2	4
URHD0025	1		1
M. vulneris	1		1
M. xenopi		1	1
Partially identified			
M. avium complex (not further identified)		5	
rapid grower		1	1
Totals	97	27	124

MAA=*Mycobacterium avium avium; WGS=Whole genome sequence*

[a]Subset of those previously reported in Pfeiffer et al. 2017.

[b]Other species determination methods included Sanger sequencing, DNA probe, high performance liquid chromatography (HPLC), and partial genome sequencing.

Table 3.2— Summary of avian taxa and species of Mycobacterium identified from 275 cases of avian mycobacteriosis at San Diego Zoo and Safari Park, 1992-2014.

Order (no. species represented)	MAA	*M. genavense*	*M.a.hominissuis*	Other spp.[a]	Unknown spp.	Total Cases
Anseriformes (23)	28			3	10	41
Bucerotiformes (3)				2[b]	4	6
Charadriiformes (3)		1			3	3
Ciconiiformes (1)		2				3
Colliformes (1)		1[b]			2	3
Columbiformes (31)	12	16	1	6	30	65
Coraciiformes (6)	1	1	1	1	5	9
Galliformes (9)	2		5	1	15	23
Gruiformes (1)	1[b]					1
Musophagiformes (3)		1			2	3
Otidiformes (1)	2					2
Passeriformes (49)	2	19	2	1	63	87
Phoenicopteriformes (2)	1			1		2
Piciformes (3)	1	1		1	1	4
Psittaciformes (12)	2	2	2	1	14	21
Strigiformes (1)					1	1
Struthioniformes (1)					1	1
Totals:	52	44	11	17	151	275

MAA=*Mycobacterium avium avium*

[a]Other species are listed in Table 1.

[b]Two different species of Mycobacterium were isolated from the same bird: *M. hassaicum* and *M. peregrinum* (*Ceratogymna atrata*) were identifeid in a black-casqued hornbill by WGS, *M. genavense* and *M. intracellulare* were identified in a Blue-naped mousebird *(Urocolius macrourus*) by WGS (Pfeiffer et al., 2017); MAA and possibly *M. xenopi* were identified in an East African gray crowned crane (*Balearica regulorum*) by DNA probe.

Table 3.3— Network centrality measures for 275 birds with avian mycobacteriosis at San Diego Zoo Global, 1992-2014. Centrality measures were calculated from the entire bird network that consisted of 16,430 birds and 905,499 edges.

	Centrality measures[a]					
	Degree			Eigenvector		
	median	IQR	p[b]	median	IQR	p[b]
All cases (n=275)	14	(6, 43)		$3.6x10^{-12}$	($1.7x10^{-15}$, $5.2x10^{-10}$)	
MAA (n=41)	37	(12, 103)	0.65	$1.3x10^{-12}$	($6.0x10^{-16}$, $4.7x10^{-9}$)	0.17
M. genavense (n=44)	45	(13, 99)		$1.3x10^{-13}$	(7.2x10-16, $1.6x10^{-10}$)	
Known genotype (n=97)	43	(12, 110)	0.20	$1.5x10^{-13}$	($6.5x10^{-16}$, $3.8x10^{-10}$)	0.047
Unknown genotype (n=178)	26	(12, 75)		$1.2x10^{-11}$	($4.8x10^{-15}$, $5.6x10^{-10}$)	

IQR=interquartile range; MAA=*Mycobacterium avium avium;*

[a]Degree=number of connected nodes; Eigenvector=the extent to which a node is connected to other highly connected nodes.

[b]Kolmogorov-Smirnov p for the equality of distributions

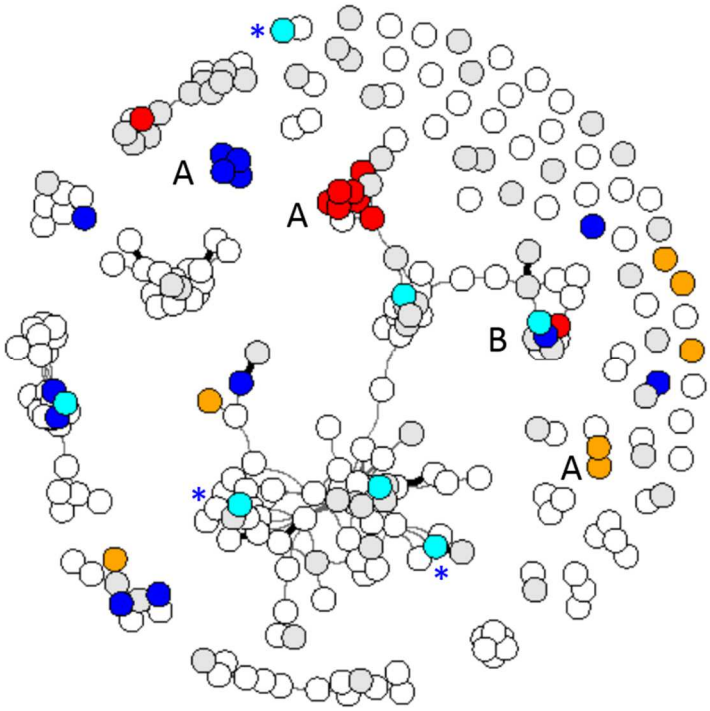

Figure 3.1— Social network of 275 birds diagnosed with avian mycobacteriosis. Each infected bird (n=275) is represented as a circle (node) and all connections between them (edges) are shown at 1° (solid line) and 2° (dashed line) of separation. For visualization purposes, the four most prevalent genotype groups, determined by comparison of whole genome sequences (WGS), are shown in colors. This included two groups of MAA (red, n=9; orange, n=7) and two groups of *M. genavense* (dark blue, n=16*; turquoise, n =7). Other known genotypes are represented in gray (n=58) and white circles denote birds with missing WGS data (n=178). Patterns of genotype groupings varied across the network. Similar genotypes clustered along paths of connectivity (e.g., A), dissimilar genotypes were found in connected birds (e.g., B), and some genotypes were dispersed throughout the network (e.g., orange, dark blue, and turquoise). *Three birds with the turquoise genotype had a multiple infection with dark blue; these are shown in turquoise with a blue asterisk (*).

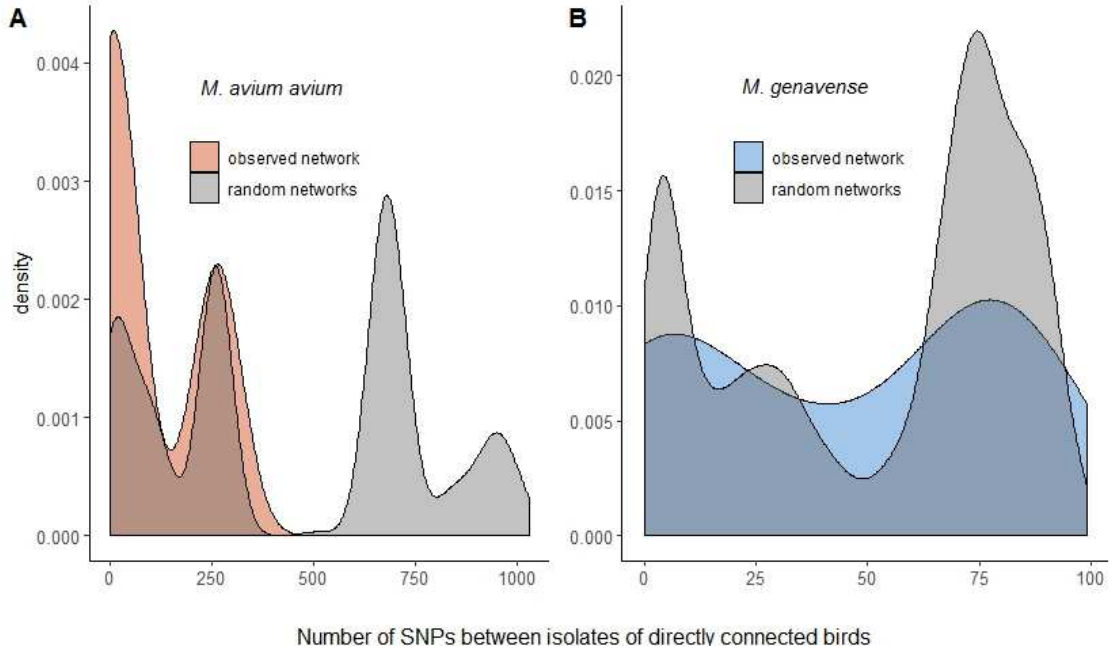

Figure 3.2— The density plots show distances between observed and random networks, where the network structure and prevalence of each genotype was preserved, but the genotype (and its corresponding genetic distance) were randomly shuffled 1000 times to other nodes to generate a random distribution of genetic distances. The genetic distance consisted of the number of identified single nucleotide polymorphisms (SNPs) that differed between the genomes of two isolates. These were evaluated separately for birds with MAA isolates and those with *M. genavense*. A. Network effects were present among ego-alter pairs of linked birds with MAA (n=26), with the observed distribution showing fewer SNPs between connected birds than expected by random (Kolmogorov-Smirnoff $p = 6.08 \times 10^{-6}$). This pattern persisted at 2° of separation for birds with MAA (n=8; Kolmogorov-Smirnoff p =0.016; data not shown). B. The distribution of SNPs between ego-alter pairs with *M. genavense* (n=20) was not different than random (Kolmogorov-Smirnoff p =0.17); data for birds with *M. genavense* were not available at 2° of separation for further evaluation.

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CHAPTER 4: LONGITUDINAL NETWORK ANALYSIS OF AVIAN MYCOBACTERIOSIS INCIDENCE IN A LARGE POPULATION OF BIRDS

ABSTRACT

Objective: To quantitatively evaluate longitudinal patterns of avian mycobacteriosis spread through a social network.

Subjects: A total of 13,409 individuals nested in a larger population of birds that were closely monitored in zoological facilities for over 22 years (1992 – 2014).

Methods: A retrospective cohort study design and social network connectivity were used to estimate the association between exposure to an infected bird and development of mycobacteriosis. Mycobacteriosis was diagnosed from histopathology and network connectivity was defined from enclosure histories over discrete time periods. Univariate and multivariable longitudinal, mixed effects logistic regression models examined whether exposure to directly-connected and indirectly-connected positive birds was associated with development of mycobacteriosis. Additional analyses evaluated associations based on characteristics of the exposure. Model assumptions were evaluated with sensitivity analyses.

Results: The main, adjusted model showed increased odds of mycobacteriosis (odds ratio or OR=2.15; 95% CI: 1.48-3.12; $p<0.001$) at the next time period (t+1), given exposure to a directly-connected bird at the current time (t) compared to those with no direct exposure. In the same model, exposure to a positive, indirectly-connected bird at time t-1 was independently associated with an increased odds of disease at time t+1 (OR=1.56; 95% CI: 1.11-2.19) compared to no indirect exposure. The associations between disease and indirect exposure persisted in risk stratified models among the subset of birds with positive indirect contacts housed in distinctly different aviaries (OR=1.61; 95% CI: 1.21-2.30) providing evidence of a contagious process. Some findings were sensitive to model variation of time divisions and initiation time.

Conclusions: The findings show that avian mycobacteriosis spread through the social network in quantifiable and discernable patterns and suggest that a contagious process is present.

INTRODUCTION

Mycobacteriosis is a well-recognized disease of birds, however patterns of natural disease acquisition are not well understood. Transmission by infected birds through the fecal-oral route is often perceived as the most important driver of disease;[1] however, environmental sources of potentially pathogenic mycobacteria may cause many avian infections. This is based on patterns of low incidence among exposed birds,[9,18,29,75] limited genetic data showing case clusters arising from multiple sources (Chapter 3),[13,81–83] and known pathways of infection for humans[6] and other species.[122,127] A challenge in separating the role of other birds and the environment in the infection process is that the transmission pathways are confounded: exposure to another bird that indirectly transmits the mycobacteria through its feces, is the same as exposure to an environment where pre-existing potentially pathogenic mycobacteria could opportunistically infect birds.

In a recent study, we used a social network analysis to disentangle the confounded transmission routes by evaluating disease clustering along different spatial and temporal network pathways (Chapter 2). Disease significantly clustered between study subjects and indirectly-connected birds that had an intermediary enclosuremate in common, but were never themselves in the same aviary. If disease spreads by a contagious process, then we would expect some of the intermediary birds who were exposed to spread the disease to other birds who had not come into contact with the originally diseased bird. The identification of significant associations between indirectly connected birds that were never in the same enclosure, strongly suggested a contagious process was present because common exposure to the same environment could not be solely driving the observed disease risk.

While disease clustering within a social network supports a role for contagion, it only tests the simple null hypothesis of whether there is a crude association. Longitudinal models that incorporate network exposure terms can more specifically test for patterns of disease incidence

over time,[136] while controlling for other potential factors, such as variability in species susceptibility, that may drive disease emergence. Different types of longitudinal models have been used to test for induction or contagion of phenomena such as smoking, obesity, and emotions in human social networks.[141,142,211]

The goal of this study was to use a longitudinal data analysis to determine whether patterns of connectivity identified through the social network determine patterns of disease incidence in a large, closely monitored population of zoo birds. We were particularly interested in whether disease is driven by not just direct exposure to infected birds (such as enclosuremates) but also by being indirectly connected to infected birds (the enclosuremates of enclosuremates) and whether there are constraints to disease spread through a social network based on the characteristics of the exposure.

METHODS

Source population and case identification

The San Diego Zoo and San Diego Zoo Safari Park (collectively referred to as San Diego Zoo Global, or SDZG) houses one of the largest and most diverse managed bird populations in the world with an average of over 3,000 birds at any time. Birds are regularly imported and exported for species propagation and move between enclosures for breeding or other management reasons. This creates a dynamic population with variation in exposure to each other and to different environments over time.

The source population included all birds that were 6 months old or older and present at SDZG for at least 7 days between January 1, 1992 and June 1, 2014. This included 16,867 birds, but 437 were removed because exposure to other birds could not be determined from housing history. The remaining 16,430 birds had near-complete, individual-level enclosure documentation

that could be used to identify periods of enclosure sharing between birds by cross-referencing enclosure move-in and move-out dates.

Birds in the source population were closely monitored by keepers and veterinarians and post-mortem examinations were performed by board-certified veterinary pathologists on any bird that died. This included histopathology on complete sets of tissues unless advanced autolysis precluded evaluation. If lesions in tissues were suggestive of avian mycobacteriosis, Ziehl-Neelsen or Fite-Faraco special stains were used to confirm presence of acid-fast bacilli (AFB). All birds with confirmed AFB in tissues were considered positive for avian mycobacteriosis, which included 275 birds from the source population, as previously reported (Chapters 2 and 3). Most of these cases were diagnosed post-mortem, however a few birds were first diagnosed from a clinical biopsy.

Network construction and identification of egos and alters

Social networks were constructed for all birds in the source population to capture heterogeneity in contact through time. Networks were assembled for 2-year time blocks starting in 1992 through the study end for a total of 12 networks, each designated as a separate time "t". Two years was considered a starting point as a sensitive measure to capture relevant, long-term exposure for most birds. It was based on previously applied estimates of maximum incubation (Chapters 2 and 3), experimental studies that document deaths 12-14 months after infection,[62,92,93] and expert opinion that incubation of infection may last for years.[14] These time periods were varied in sensitivity analyses because the distribution of the incubation period for naturally acquired infections is unknown. For each network at time t, individual birds were represented as nodes, and enclosure sharing that occurred between birds for seven days or longer was represented as edges.

Network nodes consisted of "egos", i.e. a term commonly used in social network analysis to describe study subjects,[161] and "alters", i.e. birds that were connected to the ego. Egos included the subset of birds from the source population with complete information on history of exposure to other birds. This included both birds that hatched at SDZG, as well as birds imported from elsewhere and present at SDZG for at least two years. An individual bird could serve as both an ego, as well an alter for other birds. Egos and alters were classified as negative for mycobacteriosis until the time block during which they were diagnosed as positive.

Outcome

The outcome was whether an ego became positive for mycobacteriosis at the next time, t+1. Birds that were removed from the population due to export or the end of the study, were considered negative for mycobacteriosis at the time of their removal.

Main predictors: exposure to 1° and 2° alters

Connectivity of each ego to its set of directly- (1° of separation) and indirectly- (2° of separation) connected alters was summarized from the social networks into separate, single predictors for each ego-time observation. The indirectly connected alters, also referred to as 'friends of friends' in social network analysis, are a key predictor because some of them are spatially separated from the ego and the association cannot be confounded by common exposure to the same environment. This allows for testing for the presence of a contagious process based on partitioning risk along the causal pathways illustrated in Figure 4.1.

For the main model, a dichotomous variable indicated whether the ego had any mycobacteriosis-positive 1° alters at time t (yes or no). Another dichotomous variable indicated whether the ego had any mycobacteriosis-positive 2° alters at the previous time period, t-1 (yes or no). Continuous versions of predictors (i.e., number of 1°, duration of exposure to 1° alters) were examined, but not further considered because most birds only had one directly-connected infected

alter during any given time step. Exposure to 2° alters was kept on the same scale as exposure to the 1° alters.

Additional exposure characteristics and evaluated covariates

To further isolate associations due to contagion, enclosure histories were compared between the ego at time t and its positive 2° alters to determine whether they were ever exposed to the same enclosure environment. A 3-level categorical variable classified each ego-time observation as having any positive 2° alters affiliated with the same enclosure, versus only have alters that resided in different enclosures than their ego, versus no exposure (reference group).

A categorical variable distinguished between egos that were exposed to a positive 1° alter in a small enclosure (i.e., small aviaries that included fewer than 40 birds on average) from those that only had exposure in a larger enclosure (i.e. greater than 40 birds on average; for example, large walk-through aviaries and open ponds) versus having no exposure (reference group).

Taxonomy was compared between egos and alters. Each ego-time observation was categorized as having at least one positive 1° alter of the same species versus only being exposed to a different species versus no exposure (reference group).

Infection characteristics of positive 1° alters were summarized into a single predictor for each ego-time observation according to the highest risk category regarding the presence of GI lesions, abundance of AFB in GI lesions, and cause of death. Risk categories were assigned based on a pathologist's (RP) review of histopathology, which included acid-fast stained sections of the gastrointestinal (GI) tract, representing all major areas (oral cavity, esophagus, proventriculus, ventriculus, small intestine, ceca, large intestine, and cloaca). Gastrointestinal involvement was indicated if any GI lesions were present (risk category 2) versus absent (risk category 1). Qualitative assessments of AFB abundance in GI lesions were categorized as: many (AFB were easy to find; could be diffusely present or scattered throughout lesions; risk category 3); few

(AFB were difficult to find; in two cases AFB were not observed but presumably present based on positive culture; risk category 2); or none (no AFB; risk category 1). Five cases with missing GI sections were classified as "unknown" for GI disease characterization and removed from corresponding analyses. Determination of mycobacteriosis as a cause of death was based on the distribution and severity of the mycobacterial disease and was categorized as: mycobacteriosis implicated in death (mycobacteriosis was a significant disease process with lesions that likely cause organ dysfunction; risk category 2), mycobacteriosis was an incidental finding (lesions not widely distributed and organ impairment considered unlikely; risk category 1). No exposure to any positive 1° alters at time t was the designated reference group for all variables.

Demographic and management characteristics evaluated as covariates included species, sex (male, female, unknown), import status (imported vs. hatched at SDZG), average age during time t, average age at time t grouped by quintiles, total number of enclosure moves during time t, location at time t (Zoo or Safari Park).

Analytic methods

Network descriptive statistics included the numbers of nodes, edges, and egos present at each time period, and summaries of exposure to positive 1° and 2° alters. Time-specific estimates of incidence were calculated by dividing the number of cases in egos at time t by the total number of egos present. Demographic and exposure characteristics of the bird population were summarized for the entire study period.

Screening of potential predictors of mycobacteriosis at the next time step (t+1) and estimates of unadjusted odds ratios, standard errors, and p-values were performed with single predictor mixed effects logistic regression models.[212] Models were considered with random effects for species, for individual birds, and for both. Based on the Akaike Information Criterion (AIC)[213] a model with random effects for species was chosen. Covariates identified with a p-value

of <0.20 were considered for inclusion in adjusted models. A mixed effects logistic regression model determined whether the ego's disease status in the next time block (t+1) was a function of exposure to positive 1° alters in the current time block (t) and exposure to positive 2° alters in the previous time block (t-1), while controlling for additional covariates. A stepwise forward approach was used to fit models that included the main predictors with additional covariates identified from the univariate analyses. Effect modification was evaluated by including an interaction term between the main predictors and other covariates. Functional forms of continuous covariates were determined by fitting a logit-transformed Loess curve. Competing models with similar predictors were chosen based on the AIC. Included covariates were further evaluated for statistical confounding by presence of a 10% change in coefficients of the main variables of interest when comparing models with and without each covariate, separately. Mediation by 1° alters of the association between disease outcome and exposure to infected 2° alters was assessed by comparing coefficients from models with and without the potential mediator (Baron and Kenny 1986). The final main model ("Model 1") was selected based on inclusion of the two main predictors, identification of other significant or biologically important covariates, and inclusion of random effects. This model evaluates whether disease in bird A at time t-1 spreads to bird B at time t, and then to bird C at time t+1.

Six additional models were fit to data to explore risk stratification of exposure, replacing one of the main variables in Model 1 with a categorical predictor that further characterized the exposure. Model 2 stratified exposure to 2° alters according to whether the ego had ever been housed in in the same enclosure as any of the positive alters, versus the ego was only in different enclosures, versus no exposure. Model 2 more specifically evaluates spread of disease through the network. If bird A passes the infection to B, and then B passes the infection to C as described above, then the association should be present even if A and C were housed in completely separate

aviaries. Models 3-7 stratified exposure to positive 1° alters by the enclosure size where exposure occurred (Model 3), species of the alter compared to its ego (Model 4), presence of GI lesions in the alter (Model 5), the abundance of AFB in the alter's GI lesions (Model 6), and the cause of death of the alter (Model 7).

Sensitivity analyses were performed on Models 1 and 2 using modified time blocks of 1 year, 18 months, and 3 years, each starting in 1992. A modified 2-year time block starting in 1993 was also evaluated. Models were also constructed with the subset of egos that had post-mortem data, as well as an alternative minimum observation time of four years for birds that did not hatch at SDZG.

R statistical software was used for all analyses. Networks were created with the igraph package[139] and the mixed effects logistic regression models were constructed with the glmer function in the lme4 package.[214] Statistical significance was defined as $p < 0.05$.

RESULTS

The study population included 13,409 egos (806 species), of which 203 (1.5%) were diagnosed with avian mycobacteriosis, as previously reported (Chapter 2). The median observation time for all egos was 3.4 years (interquartile range or IQR: 1.4-7 years). The ratio of males to females was similar (n=6662, 49% males; n=6291, 47% females; n=456, 3% unknown sex) and the median age on the final date in the study was 4.4 years (IQR: 1.4-7.0 years). More birds were housed at the Zoo (n=7814; 58%) than the Safari Park (n=5595; 42%) and the majority (n=8991; 67%) were hatched at SDZG versus being imported (n=4418; 33%). Birds in this population moved between the targeted enclosures on average 4.4 times (SD=4.1) during their follow-up period.

Cumulative incidence of mycobacteriosis was estimated at 1.5% for the entire study period, and was less than 1% (range: 0.19% – 0.69%) within any 2-year time block. Within these

time blocks, social networks ranged in size from 3130 to 4986 nodes and contained up to 300,000 unique edges. The median number of 1° alters per ego (i.e., "degree centrality") across all time blocks was 55 (interquartile range or IQR: 10-64). Network descriptions are summarized in Table 4.1.

Factors that met screening criteria for inclusion in multivariable models included age, location, import status, and whether the bird moved between enclosures (Table 4.2). Single predictor mixed effects logistic regression models showed highly significant associations ($p<0.001$) between exposure to a positive 1° alter and development of mycobacteriosis across all risk stratification groups (Table 4.3). Variables quantifying exposure to a positive 2° alter were significant ($p=0.008$) or approached significance when stratified at the enclosure level ($p=0.054$; Table 3).

The main multivariate logistic regression model ("Model 1"; Table 4.4), including a random effect to account for correlation across birds of the same species, controlled for all four of the covariates identified by screening. Age had a non-linear association with the outcome that showed increasing disease odds with age lessened as the birds grew older. Despite showing marginal overall significance in the adjusted model ($p=0.112$), age was included as a 5-level categorical variable and retained in the final model due to its importance as a predictor of avian mycobacteriosis.[1,7,14] For the main predictors, the adjusted model showed a significant 2.15-fold increase (95% CI: 1.48-3.12) in the odds of developing mycobacteriosis among birds exposed to a positive 1° alter at time t compared to those that were not. The odds of disease at time t+1 was 56% higher (OR=1.56; 95% CI: 1.11-2.19) given exposure to a positive 2° alter at time t-1 compared to no exposure.

Risk stratification of the main predictors (Table 4.5), showed a 61% (OR=1.61; 95% CI: 1.12-2.30) increase in the odds of disease (95% CI: 1.12-2.30) for birds connected to a positive 2°

alter that never shared the same enclosure when compared to no exposure (Model 2). The other finding from stratified models was effect modification by enclosure size. A significant 2.24 increase in odds of mycobacteriosis (95% CI: 1.19-4.24) was estimated for egos exposed to a positive 1° alter in a small enclosure compared to a large enclosure. Risk of mycobacteriosis from exposure occurring in a large enclosure was similar to no exposure (p=0.265; Model 3). Odds ratios in risk-stratified models were higher for some groups than others (e.g., exposure to a 1° alter of the same species versus a different species in Model 4; exposure to a 1° alter with GI lesions in Model 5) and statistically significant compared to the reference group of no exposure, but the contrasted levels were not statistically different from each other even without a correction for multiple comparisons.

Sensitivity analyses (Table 4.6) showed that adjusted associations between disease and having a positive 1° alter were robust to variation in the time block and subsets of the population. Associations at 2° of separation were sensitive to the length and division of the time block and were only significant in the original 2-year time-period. The other sensitivity analyses (i.e., birds with post-mortem exams and those that had longer observation periods) showed results similar to Models l and 2 (Table 4.6).

DISCUSSION

Findings from this study suggest that avian mycobacteriosis spreads through a social network in quantifiable and discernable patterns that can be detected over time. Direct exposure to an infected bird was a significant predictor that doubled the odds of developing disease at the next time period compared to those with no exposure (OR=2.15, 95% CI: 1.48-3.12). This estimate was similar across all evaluated time blocks, which supports a sustained, increased risk of disease given exposure to positive birds and where they were housed. Similar associations have been previously described in this population (Witte 2008, 2010, Chapter 2) and are expected

if the infection is being transmitted between birds. However, this finding cannot rule out the acquisition of mycobacteria from environmental sources. The environment is a source of non-tuberculous mycobacterial infections in humans[6] and other animals.[122,127] Spatial and temporal case clustering attributed to different mycobacteria has been reported in this population and environmental sources were suspected.[13] Reviews on avian mycobacterial disease also discuss the importance of potentially pathogenic mycobacteria in the environment for avian infections.[7,53]

To further test for contagion, we evaluated associations between disease and exposure to indirectly connected birds using the 'friends of friends' approach previously described (Chapter 2), building on the concept that contagious phenomena cluster along pathways of connectivity in social networks.[170] This approach uses the network structure to spatially and temporally separate the indirectly connected birds that were never housed in the same location as the ego. We found a significant 61% increase in odds of developing mycobacteriosis given exposure to a positive 2° alter (OR=1.61, 95% CI: 1.12-2.30; Model 2) that was housed in a completely separate location. This association between indirect exposure and disease is a strong indication that a contagious process is present (Chapter 2). Homophily (i.e. connected birds tend to be more alike) may also drive associations in social networks,[174] but we found no strong evidence for it in this population (Chapter 2). Effects of homophily are further accounted for by the inclusion of species as random effects in multivariable models that control for age, import status, location, and movement history.

While the overall results support that a contagious process is present, the association between disease and exposure at 2° of separation was not robust to changes in the initiation date and length of time block (Table 4.6). The 2-year time block was chosen to capture long-term, bird-to-bird exposure and the sustained viability of mycobacteria that can remain in the environment for as long as four years.[63] It captured direct exposure occurring between one day

and up to four years prior to diagnosis, depending on when birds were present in the population. It is unlikely the observed association between disease and exposure at 2° in the main model was a spurious finding due to similar associations in our previous studies (Chapters 2 and 3). It is possible that the current analysis identifies limited transmission events in network substructures, which would support mycobacteriosis being contagious, but not highly transmissible. The 2-year time block may also be the best tradeoff between increased statistical power from longer observation periods with more birds, and diminished effect by including unrelated exposures. Improved understanding of the incubation periods would aid in the development of models with better precision for capturing transmission.

The association between exposure to infected, indirectly-connected birds and disease outcome should, theoretically, be mediated by directly connected birds based on the causal pathways illustrated in Figure 4.1. In the final regression models, a mediation effect was not identified because no change was observed in the coefficient for exposure to positive 2° alters when comparing models with and without the potential mediator (data not shown). Lack of a mediation effect in these data could be the result of misclassification of the true, but unknown, pathways of transmission, other unmeasured confounding, or measurement error as described by Vanderweele.[215] While estimates of the indirect effects of mediation are beyond the scope of the current analyses, more in-depth evaluations (reviewed by Vanderweele[215]) may provide insight into understanding the causal pathways related to disease transmission between birds.

Age, import status, recent movement, and location (Zoo vs. Safari Park) were included in the models as independent predictors of mycobacteriosis with estimates shown in Table 4.4. These factors are not biological mediators of the association between the network exposure and disease outcome and there was no empirical evidence of confounding. Age is a well-documented, risk factor for disease where experimental and observational studies have shown higher infection

rates in young birds compared to older birds (reviewed by Feldman[14])[66,216] and a higher risk of developing disease when exposed at an early age.[75] However, diagnosis of disease occurs more often in adult birds,[9,38,39] which can be attributed to a longer follow-up period that provides more time for clinical expression of a slowly progressive disease.[1,7] Our findings in the present study are consistent with the literature on both accords and showed an increasing odds of disease with increasing age but the effects lessened as categorical age increased. Import status and recent movement between enclosures were also factors included in the models and have been previously identified as significant predictors of mycobacteriosis in case-control studies.[9,75] It was speculated that the strong associations may be due to stress from shipping or movement, or might be an indicator for other unknown causal factors or confounders, e.g., more susceptible species may be disproportionately imported or moved. In the present study, species effects were tightly controlled in the analysis, and imported birds were limited to those present for longer periods with more complete exposure history. The mechanism for increased risk of mycobacteriosis based on importation and animal movement remains unknown, but stress may still play a role (reviewed in Tell et al.[7]). The fourth factor included in models was location, which controlled for unmeasured differences across the two facilities. The higher risk of mycobacteriosis identified in birds housed at San Diego Zoo compared to the San Diego Zoo Safari Park may emphasize differences in environmental factors or certain microclimate niches preferred by mycobacteria, which have been reviewed elsewhere.[5,6]

The use of enclosure sharing as a proxy for true exposure does not capture heterogeneity of individual bird interactions, behaviors, or lesion presentation that may be important for infection spread, but instead approximates the potential for interaction on a population level. Therefore, additional models (Model 3 - Model 7) were constructed where direct exposure was stratified based on the assumptions that bird-to-bird infection would be passed between birds with

greater contact (i.e., same species, more restricted habitat) or those exposed to probable shedders (i.e., birds with GI lesions and advanced-stage disease). Birds in small enclosures had a significant 3.1-fold increase in odds of disease ($p<0.001$), compared to no exposure; and a significant 2.2-fold increase in odds of disease ($p=0.012$) compared to birds housed in large enclosures. Being housed in a small enclosure was previously identified as a risk factor for mycobacteriosis in this population[75] and highlights the importance of close contact in a more confined space for increased transmission potential. The magnitude of the OR estimates tended to be higher in other high-risk exposure groups, such as exposure to a positive conspecific and to birds with GI lesions, but sparse data likely decreased the statistical power to detect a significant difference between the higher- and lower-risk groups.

The data included a large population of birds with near-complete information on connectivity over time, health monitoring of all birds, and post-mortem exams with disease outcomes on almost every bird that died. These data are unparalleled in terms of completeness for network analysis as they are unlikely to be affected by some of the biases that commonly affect network data such as node censoring[198] or network boundary specification.[136] However, misclassification of network nodes or edges could still occur. If an infected bird left the population before it was diagnosed or the study timeline did not capture relevant exposure prior to import, exposure and outcome could be misclassified for some birds. To address these concerns, analyses were performed insubsets of birds with post-mortem data (n=4400 egos; 176 of them became cases) and those that were either present for the entire study period (i.e., hatched at SDZG) or were imported and observed for a minimum of 4 years (n=9554 egos; 140 of them became cases; Table 4.6). Measures of associations in both subsets were similar to Models 1 and 2 and significance of the association with 2° alters remained.

Longitudinal regression models are advantages for evaluating causal factors with a temporal sequence in observational epidemiologic studies.[217] They have also been used to determine whether the spread of phenomena over a network is associated with exposure to other nodes or characteristics of individuals.[141,142,211] To implement the model in the present study, network connections and node characteristics were measured and summarized for each ego at multiple time points and the time the outcome occurred was documented, as recommended for isolating induction or contagion in social networks.[136] Controlling for species using random effects as well as adjusting the model for age, location, import status, and movement history controlled for confounding and homophily.[174] While the current model with discrete time periods facilitated identification of 2° alters, it imposed assumptions on disease incubation and abstracted over variation in characteristics of ego-alter pairs. The model also required egos to be present across three time periods (t-1, t, and t+1) to capture exposure and disease outcome, which may limit the generalizability of findings to longer-lived species or healthier individuals. Alternative model forms, that consider time on a continuum and include network edge-level variability may further improve understanding of disease epidemiology.

The present study is one of the largest studies of avian mycobacteriosis in a well-characterized cohort ever performed. The social network approach allowed us to reconstruct connectivity between birds in the dynamic population to specifically test whether disease patterns arise from prior direct or indirect connectivity to other infected birds. Our findings showed that discernable patterns of disease were detected over time across the social network with both directly- and indirectly-connected birds. This supports the idea that a contagious process contributes to at least some infections in this population and corroborates our previous findings (Chapters 2 and 3). However, the lack of robust associations in some of the analyses suggests

that the disease transmissibility may be variable and intermittent, which further suggests that recommended interventions to remove exposed birds or prevent contact[1,7] may not be warranted.

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Table 4.1— Summary of social network properties, incidence of avian mycobacteriosis, and exposure over time among 16,430 birds at San Diego Zoo Global facilities, 1992 – 2014.

		Network characteristics				Exposure at 1° of separation	Exposure at 2° of separation	Disease in egos	
Time block	Years	N nodes (all egos and alters)	N edges	N egos	N positive egos and alters	% egos with at least one positive 1° alter	% egos with at least one positive 2° alter at previous t	N positive egos	Cumulative incidence (N positive/ N egos)
1	1992-1993	4986	226,005	3485	31	21%	--	1	--
2	1994-1995	4350	202,171	4076	28	13%	48%	24	0.59%
3	1996-1997	4407	227,097	4026	23	16%	37%	20	0.49%
4	1998-1999	4649	301,854	4242	10	11%	35%	8	0.19%
5	2000-2001	4616	262,667	4341	26	9%	16%	18	0.41%
6	2002-2003	4034	174,819	3871	30	18%	28%	22	0.56%
7	2004-2005	4272	202,180	4129	33	22%	43%	29	0.69%
8	2006-2007	4132	207,411	4008	20	9%	50%	18	0.45%
9	2008-2009	3968	185,080	3850	27	15%	34%	25	0.64%
10	2010-2011	3982	197,588	3869	16	5%	40%	14	0.36%
11	2012-2013	4089	202,895	3662	19	21%	41%	15	0.41%
12[a]	2014	3130	120,942	2766	12	--	--	9	--
Cumulative		16,430		13,409	275	29%	68%	203	1.51%

N=number; IQR=interquartile range; GI=gastrointestinal

[a]Time period 12 is truncated due to the study ending on 6/1/2014. Birds still in the population at that time were followed for 6 more months (until 11/28/2014) to obtain information on disease outcome or censoring.

Table 4.2— Single predictor mixed logistic regression models that screen for demographic and management factors to include multivariable models. Random effects were included for species (n=799) in all models. Models include 32,976 repeated measures over time for 11,374 study birds at San Diego Zoo Global facilities, 1992-2014.

Potential covariates	n cases (198[a] ego-time observations)	n noncases (32,778 ego-time observations)	Association with mycobacteriosis at time t+1		
			OR	95%CI	p
Age per year, mean (SD)[b]	6.91 (5.32)	6.77 (7.09)	1.07	(1.04-1.10)	<0.001
Age (categorical)					<0.001
0 to < 10 months	13	5804	1.00	--	--
10 months to < 3 years	34	6381	2.67	(1.36-5.27)	
3 year to < 6 years	53	6812	4.79	(2.47-9.28)	
6 years to < 11 years	57	6908	6.49	(3.34-12.61)	
≥ 11 years	41	6873	7.80	(3.82-15.91)	
Sex[c]					
male	105	16,745	0.97	(0.72-1.30)	0.830
female	93	15,431	1.00	--	--
Location					
San Diego Zoo	139	18,121	1.54	(1.06-2.26)	0.025
San Diego Zoo Safari Pa	59	14,657	1.00	--	--
Imported					
yes	153	16,007	3.77	(2.61-5.44)	<0.001
no	45	16,771	1.00	--	--
Moved enclosures					
yes	132	16,446	1.44	(1.05-1.99)	0.024
no	66	16,332	1.00	--	--

CI=confidence interval; n=number; OR=odds ratio; t=time

[a]A total of 203 ego cases were identified during the study period. Five birds that became a case are not included in the total count because they were not present across multiple time periods to evaluate exposure at time t with outcome at time t+1.

[b]Mean and SD are shown for continuous predictors

[c]Excludes 602 ego-time observations where sex was unknown.

Table 4.3— Single predictor mixed logistic regression models evaluating the longitudinal association between exposure at 1° and 2° of separation and development of mycobacteriosis at time t+1 for birds housed at San Diego Zoo Global facilities, 1992-2014. Random effects were included for species.[a]

			Association with disease at time t+1		
Exposure to positive 1° alters at time t	**Cases[b]**	**Non-cases**	**OR**	**95% CI**	**p**
Exposure to any positive alter					<0.001
yes	59	5226	2.12	(1.49-3.02)	
no	139	27,552	1.00	--	
Species of positive alters					<0.001
at least one alter was the same species	23	737	3.12	(2.07-4.70)	
all alters were a different species	36	4489	1.25	(0.73-2.11)	
no exposure	139	27,552	1.00	--	
Enclosure size where exposure to positive alters occurred					<0.001
exposed in a small enclosure (<40 birds on average)	38	1681	2.78	(1.81-4.27)	
all exposures occurred in large enclosures	21	3545	1.57	(0.95-2.59)	
no exposure	139	27,552	1.00	--	
GI infection status of alters					<0.001
exposed to at least one alter with GI lesions	34	1834	3.74	(2.56-5.46)	
exposed only to alters without GI lesiosn	25	3340	1.51	(0.99-2.32)	
no exposure	139	27,604	1.00	--	
GI AFB abundance in alters					<0.001
maximum GI abundance category of all alters was "many"	24	1329	2.85	(1.72-4.70)	
maximum GI abundance category of all alters was "few"	10	505	2.63	(1.29-5.38)	
exposed to at least 1 positive alter, but none had GI lesions	25	3340	1.56	(0.95-2.54)	
no exposure	139	27,604	1.00	--	
Cause of death in alters					<0.001
mycobacteriosis was implicated as a cause of death	47	4100	2.82	(1.46-5.44)	
all alters had incidental lesions; mycobacteriosis not the cause of d	12	1117	2.00	(1.37-2.92)	
no exposure	139	27,561	1.00	--	
Exposure to positive 2° alters at time t-1					
Exposure to any positive 2° alter					0.008
yes	68	14,772	1.58	(1.13-2.23)	
no	108	14,857	1.00	--	
Enclosure of positive 2° alter					0.054
any alters with same enclosure	25	3366	1.37	(0.82-2.27)	
only alters with different enclosures	83	11,406	1.65	(1.16-2.36)	
no exposure	68	14,857	1.00	--	

CI=confidence interval; OR=odds ratio; t=time

[a]The total study population included 13,409 individuals and 806 species. Evaluations at 1° of separation included 11,374 birds with 32,976 ego-time observations that were present across multiple time periods to evaluate exposure at time t with outcome at time t+1; 799 different species were included as random effects. Exposures at 2° of separation included 29,805 ego-time observations from 10,356 birds present across three time periods (t-1, t, and t+1) with 730 different species included as random effects.

[b]A total of 203 ego cases were identified during the study period; 198 of these were included in analyses with 1° alters because they were present across multiple time periods to evaluate exposure at time t with outcome at time t+1. Associations with 2° alters included 176 cases to evaluate exposure at time t-1 with outcome at time t+1.

Table 4.4— Main mixed effects logistic regression model estimating the adjusted association between exposure to positive 1° alters in the current time block (t) and exposure to a positive 2° alter in the previous time block (t-1), and the association with mycobacteriosis at time t+1. The model includes 10,356 birds at San Diego Zoo Global facilities represented by 29,805 repeated measures from 1992-2014. Random effects were included for species (n=730).

	Association with mycobacteriosis at time t+1				
Main Model: "Model 1"	Coefficient	SE	OR	95% CI	p
Exposed to a positive 1° alter at time t (yes v. no)	0.76	0.19	2.15	(1.48-3.12)	<0.001
Exposed to a positive 2° alter at time t-1 (yes v. no)	0.44	0.17	1.56	(1.11-2.19)	0.011
Housed at the Zoo (vs. Safari Park)	0.51	0.21	1.66	(1.11-2.49)	0.014
Imported (yes v. no)	0.982	0.22	2.67	(1.72-4.14)	<0.001
Moved to a different enclosure at time t (yes v. no)	0.512	0.17	1.67	(1.19-2.35)	0.003
Age					0.112
0 to < 10 months	-	-	1.00	-	
10 months to < 3 years	0.30	0.37	1.35	(0.66-2.78)	
3 year to < 6 years	0.65	0.37	1.92	(0.93-3.95)	
6 years to < 11 years	0.79	0.38	2.20	(1.04-4.64)	
≥ 11 years	0.93	0.41	2.53	(1.14-5.61)	

OR=odds ratio; SE=Standard error; CI=confidence interval; t=time

Table 4.5— Results of 7 separate longitudinal, mixed effects logistic regression models examining the association between listed exposures and avian mycobacteriosis at time t+1 among 10,356 birds (29,805 ego-time observations) at San Diego Zoo Global facilities from 1992-2014. All models contain a random effect for species (n total=799) and control for location (Zoo or Safari Park), import status (yes or no), whether the bird recently moved (yes or no), and age categorized by quintiles.

	Association with mycobacteriosis at time t+1					
Model main effects/levels	Coefficient	SE	OR	95% CI	p[b]	
Model 1[a]: Exposure to positive 1° and 2° alters						
Exposed to a positive 1° alter at time t (yes v. no)	0.76	0.19	2.15	(1.48-3.12)	<0.001	
Exposed to a positive 2° alter at time t-1 (yes v. no)	0.44	0.17	1.56	(1.11-2.19)	0.011	
Model 2: Risk stratification by positive 2° alter's enclosure history						
Exposed to positive 1° alter at time t (yes v. no)	0.77	0.19	2.17	(1.49-3.16)	<0.001	
Enclosure of positive 2° alter at time t-1					0.034	
ever housed in same enclosure as ego	0.34	0.26	1.40	(0.84-2.33)		
only housed in different enclosures than ego	0.48	0.18	1.61	(1.12-2.30)		*
no exposure	--	--	1.00	--		*
Model 3: Risk stratification by enclosure size where exposure to a positive 1° alter occurred						
Enclosure of positive 1° alter at time t					<0.001	
small (houses fewer than 40 birds)	1.12	0.23	3.06	(1.96-4.77)		*†
large	0.31	0.28	1.36	(0.79-2.34)		†
no exposure	--	--	1.00	--		*
Exposed to a positive 2° alter at time t-1 (yes v. no)	0.39	0.18	1.48	(1.05-2.08)	0.027	
Model 4: Risk stratification by conspecific status of the positive 1° alter						
Species of positive 1° alter at time t					<0.001	
at least one alter was the same species	1.20	0.32	3.31	(1.77-6.18)		†
all alters were a different species	0.62	0.22	1.86	(1.22-2.84)		*
no exposure			1.00	--		*†
Exposed to a positive 2° alter at time t-1 (yes v. no)	0.46	0.17	1.58	(1.12-2.23)	0.009	
Model 5: Risk stratification by lesion distribution of the positive 1° alter						
1° alter GI infection status at time t					<0.001	
exposed to at least one alter with GI lesions	0.95	0.24	2.58	(1.62-4.11)		*
exposed only to alters without GI lesions	0.59	0.26	1.80	(1.08-3.01)		†
no exposure	--	--	1.00	--		*†
Exposed to a positive 2° alter at time t-1 (yes v. no)	0.42	0.18	1.52	(1.08-2.14)	0.018	
Model 6: Risk stratification by AFB abundance in GI lesions of the positive 1° alter						
1° alter GI AFB abundance at time t					<0.001	
maximum GI abundance category of all alters was "many"	0.92	0.27	2.50	(1.46-4.28)		*
maximum GI abundance category of all alters was "few"	1.02	0.40	2.78	(1.28-6.03)		†
exposed to at least 1 positive alter, but none had GI lesions	0.59	0.26	1.81	(1.08-3.03)		‡
no exposure	--	--	1.00	--		*†‡
Exposed to a positive 2° alter at time t-1 (yes v. no)	1.51	0.41	1.52	(1.07-2.14)	0.019	
Model 7: Risk stratification by cause of death of the positive 1° alter						
1° alter cause of death at time t					<0.001	
mycobacteriosis was implicated as a cause of death	0.72	0.20	2.05	(1.33-5.36)		*
all alters had incidental lesions; mycobacteriosis not the cause of death	0.98	0.36	2.67	(1.38-3.06)		†
no exposure	--	--	1.00	--		*†
Exposed to a positive 2° alter at time t-1 (yes v. no)	0.43	0.18	1.54	(1.09-2.17)	0.014	

AFB=acid-fast bacilli; AIC=Akaike's information criterion; CI=confidence interval; GI=gastrointestinal; OR=odds ratio; t=time

[a]Model 1 was used to identify covariates and is the main model of interest. The full model is shown in Table 4.4.

[b]Estimates were compared between the different levels of multi-level variables; significant differences are indicated by the same symbol (*†‡). For Model 4, the adjusted OR for disease given exposure to a positve 1° alter in a small enclosure versus a large enclosure was 2.24 (95%CI: 1.19-4.24; p=0.012).

Table 4.6— Results of sensitivity analyses Models 1 and 2 that evaluate the association between the listed exposure and mycobacteriosis outcome at time t+1. All models include a random effect for species and control for location (Zoo or Safari Park), import status (yes or no), whether the bird recently moved (yes or no), and age categorized by quintiles. Significant associations (Type 3 p<0.05)[a] are colored gray.

	Main Model[b]		Time block variant								Subset with post-mortem data[g]			Modified minimum time present[h]		
			1 year[c]			18 months[d]		3 years[e]			2 years, shifted[f]					
Model main effects/levels	OR	95% CI	OR	95% CI		OR	95% CI	OR	95% CI		OR	95% CI		OR	95% CI		OR	95% CI	
Model 1: Main model; Exposure to positive 1° and 2° alters																			
Exposed to a positive 1° alter at time t (yes v. no)	2.15	(1.48-3.12)	1.84	(1.23-2.75)		2.09	(1.43-3.06)	2.4	(1.60-3.61)		1.91	(1.28-2.85)		2.40	(1.66-3.49)		2.04	(1.34-3.09)	
Exposed to a positive 2° alter at time t-1 (yes v. no)	1.56	(1.11-2.19)	0.80	(0.56-1.15)		1.11	(0.79-1.54)	1.16	(0.77-1.77)		0.82	(0.58-1.17)		1.52	(1.08-2.14)		1.51	(1.03-2.19)	
Model 2: Risk stratification by positive 2° alter's enclosure history																			
Exposed to positive 1° alter at time t (yes v. no)	2.17	(1.49-3.16)	1.85	(1.24-2.77)		2.08	(1.42-3.04)	2.43	(0.16-3.64)		1.90	(1.27-2.83)		2.42	(1.67-3.53)		2.06	(1.36-3.12)	
Enclosure of positive 2° alter at time t-1																			
ever housed in same enclosure as ego	1.40	(0.84-2.33)	1.11	(0.63-1.95)		0.99	(10.68-1.43)	1.03	(0.58-1.82)		0.77	(0.52-1.13)		1.38	(0.83-2.31)		1.35	(0.77-2.36)	
only housed in different enclosures than ego	1.61	(1.12-2.30)	* 0.71	(0.47-1.07)		1.45	(0.91-2.32)	1.21	(0.79-1.85)		0.96	(0.58-1.58)		1.56	(1.09-2.23)	*	1.55	(1.04-2.31)	*
no exposure	1.00	--	* 1.00	--		1.00	--	1.00	--		1.00	--		1.00	--	*			*

AFB=acid-fast bacilli; CI=confidence interval; GI=gastrointestinal; OR=odds ratio; t=time

[a]Estimates were compared between the different levels of multi-level variables; significant differences are indicated by the same symbol (*).

[b]Main model with 2 year time blocks contain 10,356 egos, 29,805 ego-time observations, and 730 species (random effects).

[c]Time block variant - 1 yr: Time blocks intervals were modified, where t=1 year, starting on 1/1/1992. The fitted model has 12,523 egos, 62,579 ego-time observations, and 800 species (random effects).

[d]Time block variant - 18 mos: Time block intervals were modified, where t=18 months, starting on 1/1/1992. The fitted model has 11,097 egos, 39,274 ego-time observations, and 764 species (random effects).

[e]Time block variant - 3 years: Time block intervals were modified, where t=3 years, starting on 1/1/1992. The fitted model has 8526 egos, 17,852 ego-time observations, and 675 species (random effects).

[f]Time shift variant: Time block intervals were shifted forward by 1 year to capture a modified version of 2 year time intervals, starting on 1/1/1993. The fitted model has 9558 egos, 26,777 ego-time observations, and 696 species (random effects).

[g]Subset with post-mortem data: the population was subsetted to birds with postmortem findings. The fitted model has 4400 egos, 13,137 ego-time observations, and 630 species (random effects).

[h]Modified minimum time present: the population was subsetted to birds that were either hatched in the population or those that were imported and present for at least 4 years. The fitted model has 9554 egos, 28,623 ego-time observations, and 681 species (random effects).

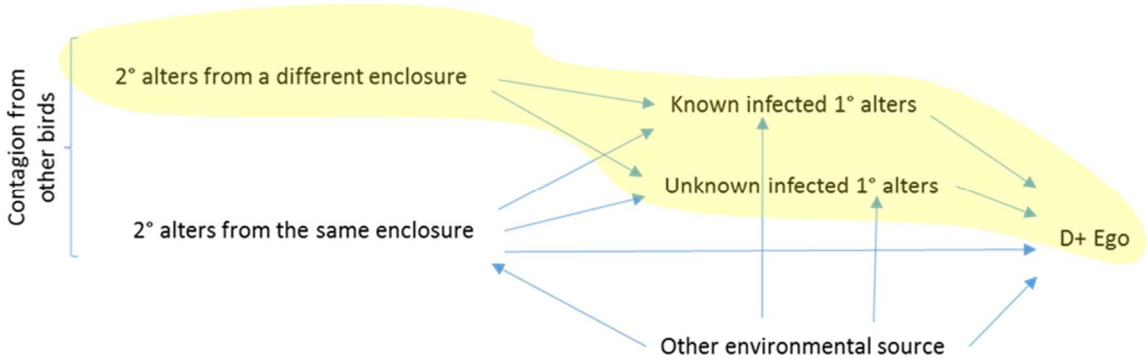

Figure 4.1— A diagram of the hypothesized causal pathways for avian mycobacteriosis. The two sources of infection include mycobacteria from environmental sources and contagious spread from other birds by primarily the fecal-oral route. The component of disease acquisition from other environmental sources remains largely unmeasured and confounds the association between exposure to 1° alters and disease, as well as 2° alters that were housed in the same aviary, but at a different time. The present study evaluates measurable exposures over time in these causal pathways, with particular interest in isolating associations along the yellow pathway. An association between disease and exposure to 2° alters that were never housed in the same location as their ego (i.e., two birds connected through an intermediary bird) isolates probable contagion because other environmental sources cannot confound the association.

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CHAPTER 5: DISCUSSION AND CONCLUSIONS

The goal of the three studies presented in this dissertation was to determine whether avian mycobacteriosis is contagious. Each study used different quantitative methods with a social network analysis approach to evaluate patterns of disease and its relation to dynamic contact with other birds. Chapter 2 used the social network structure to disentangle confounding of transmission routes and isolate associations likely due to contagion along specific spatial and temporal network pathways. Chapter 3 examined patterns of genetic similarity in the mycobacteria between connected cases in the social network. Chapter 4 used longitudinal regression models to evaluate whether direct and indirect connectivity to infected birds predicts future disease outcome. The three major conclusions and scientific advancements from this body of work are described below, a disease transmission framework is proposed, priorities for future research are identified, and final comments on the utility of network analysis are provided.

MAJOR FINDINGS

A contagious process drives at least some disease emergence

Findings from all three studies support the presence of a contagious process driving at least some disease emergence, providing new scientific evidence that supports transmission. Significant spatial and temporal clustering of cases was observed with an estimated 7-fold increase in risk of mycobacteriosis ($p<0.001$) given direct exposure to an infected bird, compared to no exposure. Significance persisted when evaluating similar associations using longitudinal, mixed effects models that adjusted for other covariates and tightly controlled for potential confounders and homophily (OR: 2.15; 95% CI: 1.48-3.12; $p<0.001$; Chapter 4).[174] Additionally, the proportion of directly connected cases with genetically similar mycobacteria was significantly greater than expected based on chance for birds with MAA (Chapter 3).

The strongest evidence for a contagious process was the detection of significant associations between the disease status of egos and their indirectly-connected alters that were never housed in the same location. In the longitudinal model, exposure to one of these alters significantly increased odds of disease by a factor of 1.61, compared to no exposure (Chapter 4), while controlling for exposure at 1° of separation, species (random effects) and other covariates. In practical terms, this finding means that the disease status of an enclosuremate's enclosuremate (or a friend's friend) that is only connected loosely through another bird could predict whether a bird gets mycobacteriosis. The significant finding was present in all three studies (Chapters 2-4) and there was no strong evidence for alternative explanations, such as homophily (Chapter 2).[174]

Avian mycobacteriosis is generally thought of as a contagious disease;[1,7] however there is little evidence in the literature documenting transmission between birds in natural populations (reviewed in Chapter 1). The three studies presented provide new evidence supporting bird-to-bird transmission in natural settings, but it is still important to consider other sources of infection. The studies described in this dissertation could not directly test for infection from purely environmental sources, but the data did show evidence of case clusters being caused by different mycobacteria (Chapter 3).[12] In Chapter 3, 66% of connected pairs had a different genotype, showing that they did not pass infection to each other in many instances. . Mycobacteria are known environmental saprophytes,[5] and it is plausible that some infections in birds originate from environmental sources, similar to infections in humans[31] and other animals.[122,127]

Avian mycobacteriosis is not highly transmissible

While the data support a contagious process, they also show that the disease is not highly transmissible. The small world network structure that we identified for birds in the study population (Chapter 2) should facilitate rapid disease spread and contribute to epidemic-style

outbreaks;[196,197] however 91-96% of exposed birds never become infected.[75] In this closely monitored population, there have not been epidemics and the incidence of disease has remained low (1.5%; Table 4.1).[9] The lack of robust associations between disease and exposure to 2° alters in the longitudinal analyses further suggests that transmission between birds may be variable and intermittent.

The conclusion that avian mycobacteriosis is not highly transmissible in natural settings is consistent with other research. In his review of early literature, Feldman concluded that mycobacteria were not readily contagious and that bacteria must be given repeatedly over long periods to ensure infection.[14] More recently, researchers have questioned the transmissibility of *M. genavense* based on lack of disease among contacts.[18,29] The present studies further substantiate this idea by showing that the network structure is not preventing the spread of disease by isolating birds in enclosures, rather it should facilitate rapid dissemination, which does not occur.

Low transmissibility has important implications for disease management. Within zoos there is wide concern that birds exposed to other infected birds are likely to become subclinical carriers and eventually transmitters of the disease. Fear that these subclinical carriers will spread the bacteria to the naïve population in the same aviary has led to elaborate disease mitigation efforts. Current recommended protocols focus on breaking the bird-to-bird transmission through halted breeding, reduced movement in and out of exhibits, and depopulation.[1,38,40–43] This has a tremendous negative impact on bird management and may not be a reasonable approach for a disease with low transmissibility. Modifications of current recommendations to a more passive approach that would include population-level surveillance and good sanitation may be warranted if studies in other populations yield similar findings regarding limited disease transmissibility.

Avian mycobacteriosis is not a single disease

Whole genome sequencing revealed that many different mycobacteria are contributing to infections in this population (Chapter 3).[12] Among directly-connected pairs of cases, 66% had genetically distinct mycobacteria, indicating that infections were not being passed, rather acquired from other sources. Therefore, one should not assume that case clusters are always caused by the same mycobacteria.

The most common species identified were MAA and *M. genavense*, which is similar to other reports.[9–12,74,81] Associations consistent with a contagious process were identified for MAA, but not for *M. genavense* (Chapter 3). Several explanations for this finding were previously discussed (Chapter 3), among which is the possibility that the disease transmissibility is different for these species of Mycobacterium. Additional studies clarifying transmission mechanisms and describing genetic diversity are needed to improve understanding of the epidemiology of *M. genavense* infections. Improved methods that incorporate genetic data into outbreak investigations could reduce the negative impact of the current disease management approach on population breeding, sustainability, and reintroduction efforts.

FURTHER DISENTANGLEMENT OF TRANSMISSION PATHWAYS

Consideration of different transmission pathways is critical to implementing effective interventions and predicting disease outcomes because even minority routes may be consequential for determining disease thresholds and dynamics.[218] Infectious agents can vary from having simple, direct transmission (e.g., measles, influenza) to indirect transmission that involves multiple hosts, multiple routes, and a complex life cycle (e.g., *Toxoplasma gondii*, Rift Valley Fever Virus) (reviewed by Webster and colleagues[219]). The majority of epidemiologic theory investigating disease transmission has focused on the single host and single parasite systems. Systems with multiple modes and routes of transmission are studied less often because the direct measurement of transmission is difficult.[220] In the present studies (Chapters 2-4) a

contagious process was isolated using indirect network measures. Estimating the fraction of cases attributable to different transmission pathways (via another bird or exclusively from environmental sources) was not possible due to complete confounding of the pathways. Pathogen genetics show promise to distinguish between transmission pathways (e.g., Gardy et al.[78]). Chapter 3 used this approach to compare WGS of mycobacterial isolates, but genetic data were only available for some birds, thereby limiting the ability to identify the presence or absence of transmission events. Efforts to obtain more complete pathogen genetic data are underway and may provide future opportunities to understand the relative importance of different transmission pathways.

Mathematical models may clarify infection transmission using non-linear transmission functions, contact matrices, and networks.[220] Building on multi-host, multi-mode transmission models presented by Webster and colleagues[219], a simplified model for avian mycobacteriosis is proposed in Figure 5.1. Most avian mycobacteriosis infections presumably result from environmental contact,[1,7] irrespective of whether the pathogen originated from another bird (represented as E_1) or an independent environmental source (E_2). Deterministic or simulation models distinguishing between these two entangled, but conceptually separate transmission pathways could facilitate estimation of the relative transmission rates. Estimating the per capita transmission rates (β_{11}, β_{12}, β_{21}, β_{22}), the average duration of infection ($1/\gamma_1$, $1/\gamma_2$), as well as the rate of contamination (α_1, α_2) will be key to understanding the role of the environment versus transmission from other birds. Of note, the illustration does not capture different modes of transmission (i.e., ingestion, inhalation, or implantation into open wounds), the potential for direct transmission, changing population dynamics, or the potential for combined frequency and density-dependent transmission.[218] Additional considerations for this basic model may also be needed.

NETWORK ANALYSIS: A POWERFUL TOOL FOR INVESTIGATING AVIAN MYCOBACTERIOSIS EPIDEMIOLOGY

The present studies included a large population of birds with health monitoring, near-complete information on connectivity over time, and post-mortem exams with disease outcomes on any bird that died. These data are unparalleled in terms of completeness for social network analysis as they are unlikely to be influenced by some of the biases that commonly affect network data such as node censoring[198] or network boundary specification.[136] While associations between disease and direct connectivity have been evaluated in this population,[9,75,221] the traditional epidemiologic methods previously used could not capture the population dynamics, the variability in relationships between pairs of birds, or specifically test for contagion. The social network approach offered an alternative, powerful tool that accounted for some of the previous limitations, thereby uncovering subtle disease patterns that could be attributed to contagion.

The approach to isolating confounding of disease transmission pathways using 2^{o} of separation or 'friends of friends' and spatiotemporal partitioning of the network structure is new (Chapter 2). Inferring contagion by testing for disease clustering in parts network requires quite complete network ascertainment, very good information on location over time, and a large number of relationships between birds to create the network edges. This approach was used in all three studies (Chapters 2-4) to isolate associations that were likely explained by contagion alone.

Network analysis has not been widely applied to zoo populations, however the well-characterized data kept by zoos as part of management practices and health monitoring provides opportunities to understand disease epidemiology and other phenomena in complex systems.

PRIORITIES FOR FUTURE RESEARCH

Additional studies are needed in different populations with different management and baseline incidence rates to improve understanding on how connectedness of birds, and other factors affect the epidemiology of disease. Extensions of the current research are listed below.

- Further refinement of the distributions of the incubation and infectious periods of naturally-acquired avian mycobacteriosis could improve model assumptions.
- Research clarifying transmission mechanisms and describing genetic diversity could improve understanding of the epidemiology of *M. genavense* infections.
- Alternative forms of the network models examining effects in a time-to-event framework with edge-level attributes may help refine estimates of disease risk. Social network analysis at the level of the enclosure, rather than the bird, may also provide more insight into disease transmission.
- Agent-based simulation or deterministic mathematical models could further explore disease dynamics in a virtual population based on the identified social network and estimated parameters. Figure 5.1 offers a basic starting place for model construction.

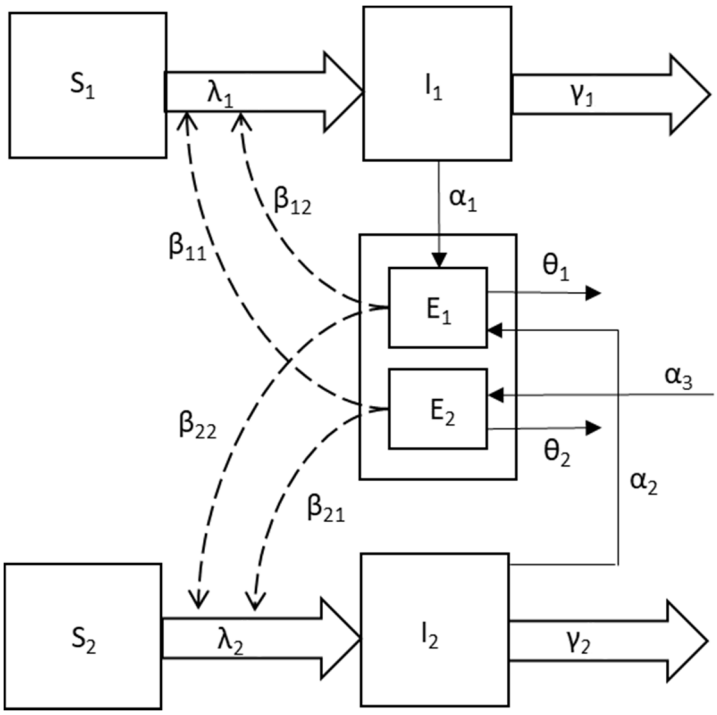

Figure 5.1— Illustration of simplified compartmental model for capturing the different transmission routes of mycobacterial infection in two species of birds housed in the same aviary. Block arrows represent the flow of individuals between compartments, dashed lines represent transmission from environmental compartments to birds.

Where:
S = susceptible
I = infected
E = infective stages in the environment,
β = per capita rate at which susceptible hosts become infected by the designated transmission route; this is a function of contact with pathogenic mycobacteria in the environment and the probability of transmission when contact occurs
$1/\gamma$ = average duration of infectiousness
α = rate of environmental contamination from infected birds (α_1, α_2) or external sources (e.g., municipal water, α_3)
θ = decay rate of infective stages in the environment

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