Salmonella INFECTION IN SOUTHEASTERN BIRDS

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Salmonella Infection in Southeastern Birds

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Abstract

Avian disease is an emerging field of research that not only addresses human health issues, but also informs conservation strategies for wild bird populations. Salmonellosis is a gastrointestinal disease caused by bacteria in the genus *Salmonella. Salmonella* infections are correlated with periods of increased avian activity such as times of migration, but questions regarding seasonal patterns of infection are far from resolved. In this study I measured *Salmonella* infections present at three feeder sites around Clarksville, TN in summer and winter to determine if there is a seasonal influence on the number of infections. My results show no evidence to support a seasonally influenced variation in the number of *Salmonella* infections. I found no evidence to suggest that any species sampled are more or less likely to be infected with *Salmonella*. The results of this study lead me to conclude that there is no species or seasonal influence on the rate of *Salmonella* infection as well as establishes a regional baseline of *Salmonella* infections at feeder stations.

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Introduction

Disease ecology in avian populations is an important emerging field of research, crucial to maintain healthy populations of wild birds in a changing world where human impacts can negatively influence wild animal populations. As well as impacting avian populations, diseases carried by avian vectors can transmit infection to human hosts through indirect contact with contaminated feces or direct contact with infected birds. Recently, major outbreaks of avian influenza have made headlines throughout the world and brought avian disease research into the forefront of fighting potential global pandemics (Fouchier *et al*, 2003). Understanding how avian transmitted diseases impact human populations is vital to control potential outbreaks since birds are highly mobile and certain species are closely associated with human settlements, leading to an increased likelihood of humans coming in contact with infected birds. Large-scale transmission of avian diseases could potentially have major negative impacts on human populations as well as avian ones. Massive avian die-offs associated with *Salmonella* have been recorded in songbird and waterfowl populations (Hall *et al*, 2008) as well as *Pasteurella multocida*, the causative agent of avian cholera, and (Botzler, 1991) *Clostridium botulinum*, whose toxin is associated with avian botulism poisoning (Rocke & Samuel, 1999). Outbreaks of these diseases can devastate local bird populations and can have lasting environmental impacts due to the ability of bacteria and associated toxins to persist in the environment for extended periods of time (Hubálek & Halouzka, 1991), putting migrating populations of birds at risk as well as other mammalian and reptilian species. Coupled with their ability to infect

and cause disease in avian hosts, the previously mentioned bacteria potentially pose a threat to human populations as well (Heddelston & Wessman, 1974, Grayson, 1988, Inderlied *et al*, 1993).

Large-scale die-offs of birds are usually associated with viruses and bacteria that are easily spread from one individual to another, such as *Salmonella* and West Nile Virus. Periods of increased bird activity, such as migration when large numbers of birds come together in flocks (Reed *et al*, 2003), and areas where greater numbers of birds are found in close proximity, such as rookeries and feeder stations, play an important role in the transmission of infectious agents (Brittingham & Temple, 1986). Once an individual at a feeder station or rookery becomes infected with a particular agent, the infection can spread rapidly to uninfected birds through contact with contaminated fluids or direct bird-to-bird contact. The ability of bacteria to persist at a feeder station outside of a host, specifically in feces, can increase the rate of infection and is important for the transmission of infection from one individual to another. A large number of bacterial infections have become associated with feeder stations, many of which have the ability to cross host species and infect humans (Brittingham *et al*, 1988). Diseases such as tuberculosis, chlamydiosis, and salmonellosis have been found to persist at feeder stations through out the year (White *et al*, 2006).

Avian tuberculosis, caused by *Mycobacterium avium* (an aerobic, non-motile, non-spore forming, acid-fast bacteria), is thought to have the ability to infect all known species of birds (Friend, 2001), especially passerines, as well as domestic livestock and humans (Ryan *et al*, 2004). Avian tuberculosis is closely associated

with captive birds, turkeys and parrots, but has also been found in wild bird populations, particularly in species closely associated with livestock yards and grains, such as European House Sparrows and European Starlings (*Sturnus vulgaris*) (Wilson, 1960). In North America, both House Sparrows (Pimentel *et al*, 2005) and European Starlings (Linz *et al*, 2007) are invasive species as well as frequent visitors to feeding stations. Infected individuals can carry avian tuberculosis to feeder stations with them, putting other birds at high risk of contracting an infection. Birds showing signs of avian tuberculosis exhibit lameness and a ruffled appearance, are often weak and lethargic and may have severe diarrhea (Dhama *et al*, 2011).

The transmission of avian tuberculosis is through fecal contamination of the environment with ingestion of contaminated food and water being the primary route of transmission (Gaukler *et al*, 2009). *Mycobacterium avium* has the ability to survive outside of a host vector for years in the environment given proper conditions (Dhama *et al*, 2011) increasing the likelihood of a few infected individuals to contaminate an area that has a prolonged avian presence, like a feeder station. Typically avian tuberculosis is not associated with large-scale die-offs of wild passerine birds but large-scale avian tuberculosis associated die-offs have been reported in domestic fowl (Wilson, 1960). Close monitoring of avian tuberculosis levels in captive and wild avian populations is crucial to controlling the spread of the bacteria to other bird populations, domestic livestock, and human hosts (Dhama *et al*, 2011).

Since *Mycobacterium avium* has the ability to infect human hosts it is important to limit the spread of avian born transmission. Birds can contaminate an

environment or water source leaving *Mycobacterium avium* in the environment for years after they discontinue use of the site (Dhama *et al*, 2011). Once *Mycobacterium avium* begins to cause disease in a human host it causes a syndrome known as *Mycobacterium avium* complex (MAC). This syndrome commonly impacts people with depressed or compromised immune systems, such as the elderly, infants, and AIDS/HIV+ individuals. It is estimated that up to 40% of advanced AIDS/HIV+ individuals in the United States are impacted by MAC at some point in their lives (CDC, 1993). MAC commonly originates from contact with *Mycobacterium avium* contaminated soil or water but can also originate from contact with infected bird feces or direct contact with infected birds and mammals (Martin & Schimmel, 2000). Once *Mycobacterium avium* enters the host, usually through inhalation or ingestion, the bacteria will begin to establish infection. The first signs of disease are commonly coughing and fever, followed by severe diarrhea if the infection is established in the gastrointestinal tract. In some cases *Mycobacterium avium* can disseminate through the patient causing anemia and malabsorption (Inderlied *et al*, 1993). Prevention of MAC involves prophylaxis antibiotics, such as clarithromycin or azithromycin (CDC, 1993). Treatment of MAC involves a number of anti-tuberculosis drugs such as rifampicin, rifabutin, ciprofloxacin, amikacin, ethambutol, streptomycin, clarithromycin and azithromycin (CDC, 1993).

Established infections of the pulmonary system that show signs more like those of human tuberculosis (*Mycobacterium tuberculosis*) are known as "Lady Windermere Syndrome", named after a character in Oscar Wilde's play *Lady Windermere's Fan*. Patients suffering from Lady Windermere Syndrome experience

a chronic cough, shortness of breath and fatigue, as well as a host of other variable symptoms (Bhatta *et al*, 2009). Treatment of Lady Windermere Syndrome usually involves a three-drug regimen of clarithromycin or azithromycin, plus rifampicin and ethambutol. Treatment typically lasts at least 12 months (Bhatta *et al*, 2009).

Along with avian tuberculosis, avian clamydiosis has become closely associated with doves and pigeons, sometimes infecting songbirds, at feeding stations. Avian chlamydiosis is caused by the bacterium, *Chlamydophila psittaci*, a gram negative, coccoid, obligate intracellular bacterium in the family Chlamydiaceae (Elías *et al*, 2013). Avian chalmydiosis has been reported in at least 30 orders of birds, and can be found worldwide, regardless of time of year (Elías *et al*, 2013). While doves (Columbiformes) and parrots (Psittaciformes) tend to be the main vector of avian chlamydiosis, (hence the species name *psittaci*) infections in finches (Fringillidae) have been recorded (Elías *et al*, 2013). Some birds carry this organism asymptomatically while others become mildly to severely ill, either immediately or shortly after they have been stressed. Significant economic losses have been seen in domestic fowl, and high mortality can occur in clinically diseased psittacines (Elías *et al*, 2013).

Transmission of avian chlamydiosis occurs through a number of different routes, respiratory, contamination, consumption, or though direct contact, just to name a few. Respiratory transmission occurs through inhalation of infectious dust or airborne particles, such as feathers or dirt, that has been contaminated with feces. Large quantities of *Chlamydophila psittaci* are excreted in feces, and can become aerosolized when the fecal material dries (Harkinezhad *et al*, 2008).

Infectious elementary bodies of *Chlamydophila psittaci* have been reported to persist for months in the environment after the initial infected host has left (Johnson *et al*, 2000).

Birds infected with avian chlamydiosis, once the disease takes hold, typically will stop eating and remain motionless on perches or feeder stations until death. Diseased individuals also exhibit a discharge from the eyes and nose and a grey to rust red diarrhea (Elías *et al*, 2013). As well as infecting avian hosts, *Chlamydophila psittaci* can cause disease in domestic livestock, mammalian pets, and humans (Johnson *et al*, 2000). Dogs and cats exhibit neurological symptoms and spontaneous abortions in horses have been attributed to *Chlamydophila psittaci* (Gresham *et al*, 1996). Humans are also easily infected by *Chlamydophila psittaci*, through contact with infected birds and contaminated feces causing a disease known as psittacosis, or parrot fever. Pandemic level disease in the USA and Europe have been associated with infections originating from imported parrots, (Harkinezhad *et al*, 2007) with the last major outbreaks occurring during late 1929 and early 1930. This outbreak resulted in a mortality rate of 19.5% among those infected (Ramsay, 2003). While global psittacosis pandemics are no longer common, small scale human infections have been linked to wild bird populations as recent as 2002 (Telfer *et al*, 2005). As many as 50 cases of human psittacosis are reported each year in the United States, although that number is thought to be higher due to incorrect diagnosis and unreported cases (CDC, 2014).

Disease in humans is closely linked with contact of infected avian hosts. In a study conducted in Australia in 1988 involving 135 psittacosis patients, 85% of

those suffering from psittacosis reported recent exposure to birds (Grayson, 1988). Although human-to-human transmission is thought to occur, it has not been proven (CDC, 1998). Once *Chlamydophila psittaci* enters a human host it has an incubation period of up to 70 days before symptoms begin to appear. Following the onset of disease, atypical pneumonia is first observed. High fevers, arthralgias, diarrhea, conjunctivitis, epistaxis and leukopenia, are commonly reported in the first few weeks of infection (Grayson, 1988). Treatment of psittacosis involves the use of tetracyclines, and antimicrobial therapy that must continue for up to two weeks after the fever breaks. Control of avian chlamydiosis is important due to the risks associated with avian populations as well as domestic livestock and humans.

Prevention of both avian tuberculosis and chlamydiosis at feeding stations involves cleaning and disinfecting both the feeder itself as well as the surrounding area to remove contaminated feces with a 10% bleach water solution at least once a week. After a full cleaning, feeders should be allowed to dry before refilling. Discarded seeds and seed husks, on and below the feeder, should be removed at a weekly interval. If avian morality is observed at a feeder, a stronger 30% bleach water solution should be used to clean the feeder and surrounding area at least three times a week and discarded seeds and husks should be removed daily. If a disease outbreak has been observed, the most effective method of control is to discontinue feeding for ten days after thoroughly cleaning the feeder and surrounding area (USFWS).

Coupled with avian tuberculosis and chlamydiosis, avian salmonellosis has recently become a disease of concern regarding feeder transmitted avian diseases.

Avian salmonellosis is caused by species of *Salmonella*. To date, approximately 2,300 different strains of *Salmonella* bacteria, also called serovars, have been identified using antigens present on the bacteria that elicit an immune response. Currently all 2,300 serovars are divided into two different species, *Salmonella enterica* and *S. bongori* (Agbaje *et al*, 2011). *Salmonella enterica* is further divided into six different subspecies based on different biochemical characteristics, resulting in a complex naming system for each serovar, such as *Salmonella enterica* serovar *typhimurium* (Agbaje *at al*, 2011). Species referenced will be named by their serovar type, such as *Salmonella typhimurium*.

All species of birds should be considered susceptible to infection by *Salmonella* and the outcome of the infection, once the disease sets in, depends on factors such as age, stress levels, and serovar virulence (Friend, 2001). Prior to the early 1980's most isolates of *Salmonella spp.* from wild birds were found in visually healthy birds, birds with a previously underlying condition, or from small-scale die-offs involving a small number of birds that had succumbed to the disease (Refsum *et al*, 2002).

Since that time period, large scale die-offs associated with wild birds using feeding stations have been reported from at least 5 different countries (Tizzard, 2004). These die-offs are usually associated with passerine birds, specifically European Starlings (*Sturnus vulgaris*; Carlson *et al*, 2011), European House Sparrows (*Passer domesticus*; Kirk *et al*, 2002) and finches (Fringillidae; Pennycott *et al*, 1998). Salmonellosis has also been associated with large-scale die-offs of several species of waterfowl (Anseriformes), gulls (Charadriiformes; Wobeser,

1981) as well as grebes (Podicepiformes; Duncan *et al*, 1983) and herons (Pelicaniformes; Locke *et al*, 1974). However large-scale die-offs in wild populations associated with *Salmonella* are rarely reported, with the exception being waterfowl, passerines, and colonial nesting species (Tizzard, 2004). Colonial nesting species, such as gulls, exhibit nestling die-offs shortly after the young are hatched during the early summer (Tizzard, 2004). The mortality rates among infected birds also varies by species (Hall & Saito, 2008). The estimated mortality rate in American Goldfinches (*Spinus tristis*) is 65.3% (Hall & Saito, 2008) of all infected birds while the estimated mortality rate of Ring-Billed Gulls (*Larus delawarensis*) is 10.8% of all infected birds (Hall & Saito, 2008). Mortality rates in other passerine species vary from 35.1% for European House Sparrows (*Passer domesticus;* Hall & Saito, 2008) to 39.2% for Brown-Headed Cowbirds (*Molothrus ater;* Hall & Saito, 2008) and become drastically higher in species such as Evening Grosbeaks (*Coccothraustes vespertinus;* Hall & Saito, 2008) where an 88.5% death rate among those infected is estimated. Mortality rates in other common feeder species vary between 58.2% for the American Cardinal (*Cardinalis cardinalis)* all the way to 83.1% (Hall & Saito, 2008) for the Pine Siskin (*Spinus pinus)* and 84.6% (Hall & Saito, 2008) for the Common Redpoll (*Acanthis flammea*).

Avian salmonellosis occurs worldwide in a large number of wild bird species and is found in a wide variety of habitats. Salmonellosis in perching birds (Passeriformes) is an emerging disease associated with urban and human impacted environments, such as landfills. Passerines are birds in the order Passeriformes. This order makes up over half of the identified birds species worldwide. These

birds, often known as perching birds, are found on every continent except for Antarctica. The order Passeriformes contains over 5,000 species and more than 100 different families of birds, making it the most diverse order of vertebrates, second only to Rodentia, the order that contains rodents such as squirrels and beavers. The order Passeriformes is divided into three suborders, Tyranni, Passeri, and Acanthisitti. Most passerine birds are quite small, with the smallest passerine, the Short Tailed Pigmy Tyrant (*Myiornis ecaudatus)*, weighing around 4.2 grams (Collias, 1997) with the largest member of the order, the Common Raven (*Corvus corax*), weighing about 1.5 kg (San Diego Bird Atlas, 2000).

While passerine and waterfowl associated salmonellosis is most common, *Salmonella* has been recently been identified in populations of birds in the Antarctic, especially Adélie Penguins (*Pygoscelis adeliae*) and South Polar Skuas (*Catharacta maccormicki*; Oelke & Steiniger, 1973) The geographic distribution of salmonellosis in wild, free ranging, birds is closely associated with environmental contamination, feeding stations, and human activity (Tizzard, 2004). Salmonellosis can present itself anytime of the year regardless of season. Passerine disease outbreaks associated with feeding stations are closely correlated with increased avian use at the feeding station.

Since the late 1800's, salmonellosis as a disease of poultry has been widely studied. Two major serovars of *Salmonella spp.* have become associated closely with poultry disease, *Salmonella pullorum* (Pullorum Disease; Friend, 2001) and *Salmonella gallinarum* (Fowl Typhoid; Friend, 2001). These two strains of *Salmonella* have received considerable attention due to their economic impacts on

the poultry industry. Wild birds have been known to be infected with these strains although they are more commonly infected with variants of *Salmonella* that are referred to as paratyphoid forms, such as *Salmonella enterica* serovar *typhimurium* and serovar *paratyphoi* (Maskey *et al*, 2006). Paratyphoid forms of *Salmonella* make up the vast majority of *Salmonella* isolates from wild birds and are becoming increasingly important due to the role they play in avian mortality (Hughes *et al*, 2008).

As well as impacting avian populations, salmonellosis in humans may present with severe symptoms. Symptoms are usually gastrointestinal in nature and include nausea, vomiting, severe stomach cramps, and bloody diarrhea. These symptoms can be life threatening in young children and the elderly and usually last for about a week after initial bacterial contact (CDC, 2012).

Salmonella is a genus of rod shaped, gram negative, non-spore forming, motile enterobacteria, that commonly ranges in length from 0.8 μm to 5 μm (Giannella, 1996). *Salmonella* was identified in 1885 by Theobald Smith who named the new genus *Salmonella* after Daniel Elmer Salmon, an American veterinary pathologist, the administrator of the lab Smith worked in at the time.

Salmonella are chemoorganotrophs, which means they obtain their energy from reactions involving organic sources and do so best in anaerobic conditions. *Salmonella* is found worldwide in both endothermic and exothermic animals as well as in the environment (Giannella, 1996). *Salmonella* are also facultative intracellular pathogens that enter the cell through the up take of solutes from the extracellular matrix. Once inside a host, *Salmonella* survives in the gastric fluids of the stomach

and travels to the small and large intestines. Once in the intestinal tract, it invades the intestinal epithelial mucosa and begins to produce toxins. Interaction between *Salmonella* and the epithelium triggers the chemotaxis of phagocytic cells to the diseased site. This cellular response involves both neutrophils and macrophages migrating to the luminal surface where they begin eradicating the bacterial pathogen (Henderson *et al*, 1999). The inflammation of the intestinal lining causes diarrhea and can lead to severe ulceration and eventually destruction of the mucosa all together. Once a lesion is formed, the bacteria can disseminate from the intestine and cause a systemic infection (Giannella, 1996).

Avian salmonellosis signs vary greatly and depend on the species and age of the bird, as well as the serovar of *Salmonella* causing the disease. Young birds often exhibit more obvious signs of salmonellosis and more outwardly pronounced signs of disease. Infection with *Salmonella* can result in an acute disease with a very rapid death or a more prolonged chronic infection with a diseased bird shedding *Salmonella* for weeks to months before death (Tizzard, 2004). Once diseases takes hold typical field signs among all species include ruffled feathers, drooping of wings, diarrhea, and severe lethargy. Diseases birds often exhibit loss of coordination, tremors and convulsions shortly before death. The vents and eyes of diseased birds often swell and stick together due to fluid discharge and blindness has been reported in some cases. Birds that carry a chronic infection often appear severely emaciated. In rare cases a small percentage of infected birds can be asymptomatic carriers of *Salmonella* and shed bacteria for the rest of their natural life without ever succumbing to salmonellosis (USFWS).

Overall, the prevalence rate of *Salmonella* in the wild is low with one study placing it at 2%-3% (Tizzard, 2004). Populations of birds that frequent feeding stations carry a higher rate of infection with one study reporting a 20%-50% infection rate (Tizzard, 2004). Control of *Salmonella* infection at feeders is accomplished by removing spilled and soiled seed on and under the feeder. Feeders should be washed with a 10% bleach water solution on a semi annual basis to eliminate bacteria on the feeder. If mortality caused by *Salmonella* is observed at a feeder, the feeder should be removed and cleaned using a bleach solution and feeding should not resume for a period of at least two weeks (USFWS). Control of *Salmonella* in avian populations is very important due to the large number of birds, up to a 50% mortality rate among those infected, that are killed by infection each year (Tizzard, 2004). Not only do infected birds pose a threat to other populations of wild birds, they also threaten the poultry farming industry and other livestock producers.

Since the role of feeding stations has been identified in the increased infection rate of *Salmonella* it is crucial to identify species that are at an increased likelihood of contracting salmonellosis. Pine Siskins (Hernandez e*t al*, 2012) are reported to be frequent carriers of *Salmonella*, along with Evening Grosbeaks, (Tizzard, 2004) European House Sparrows, (Kirk *et al*, 2002) and Brown-Headed Cowbirds, (Tizzard, 2004). Members of the finch family, such as the American Goldfinch and the House Finch (*Haemorhous mexicanus*), are also frequent carriers of *Salmonella* infection (Pennycott *et al*, 1998).

While a lot is known about avian salmonellosis, there are a number of things that are unknown in regards to this disease. Although it is documented that *Salmonella* is highly associated with increased avian use of feeding stations (Tizzard, 2004) and can present at anytime of the year, it is unclear if there is a specific period of the year when *Salmonella* infection risk is highest. This study aims to investigate that question by determining if there is a difference between infection rate for the summer and winter seasons as well as establishing a baseline infection rate. This study also aims to discover which family or species of birds is more likely to be carriers of *Salmonella*. While the current body of literature places finches high on the list of species likely to carry *Salmonella* (Tizzard, 2004), this study aims to determine if any other species are likely to carry the infection. Finally, this study intends to determine if any species that are identified as carriers of *Salmonella* infection were previously unknown to carry the infection.

Methods & Materials

I. Collection of Birds

A. Study Species

Birds that are frequent visitors to feeding stations were selected for this study due to the role they play in the transmission of *Salmonella*. Common orders of birds found at feeding stations including Passeriformes, Columbiformes, and Piciformes were tested for *Salmonella*.

B. Trapping Conditions

Methods for trapping and handling birds were approved by the APSU IACUC committee (Protocol #13.009). Three trapping locations were selected in Montgomery County, Tennessee with established feeding stations that had been continuously operating for a time period greater than six months. Trapping took place in both the summer and winter season, with the summer season ranging from May to August and winter from December to March. Six trapping sessions were conducted during the summer season and four trapping sessions were conducted during the winter season. Once permission had been granted from the homeowner, a mist net was erected directly in the flight path of birds that were coming to feed at the feeding station. Only birds that were captured arriving or departing from the feeding station were tested for *Salmonella* infection.

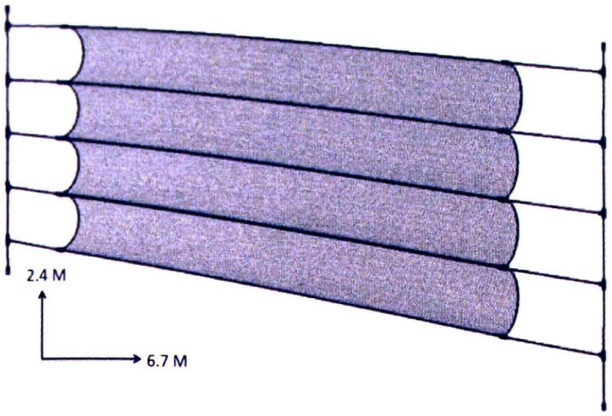

Figure 1.0 Dimenstions and Diagram of a Mist Net (Picture from Modern Falconry, 2014).

Once a bird had become entangled in the mist net it was carefully extracted and placed in a clean cloth bag until it was removed for testing. Each cloth bag was only used once to eliminate the possibility of fecal cross contamination. After a bird had been removed from the bag, measurements including weight, wing and tail length, age, and sex were recorded, along with the species, as well as any noticeable defects. After data collection, a uniquely numbered USGS aluminum identification band was placed on the right leg of each bird and the band number recorded. A collection number of 50 birds was established for each season, summer and winter, for a total of 100 birds for the entire year. This number was chosen because it was felt to give a large representative sample of common feeder birds. Birds that had been previously banded, if recaptured, were not retested during the same season to limit the possibility of counting the same infected individual twice in our data analysis.

II. Bacteria Collection and Enrichment

A. Bacteria Collection

After data collection, a cloacal swab was taken from each bird. A small, sterile cotton swab was dipped in a capped test tube of sterile water. This wetting process

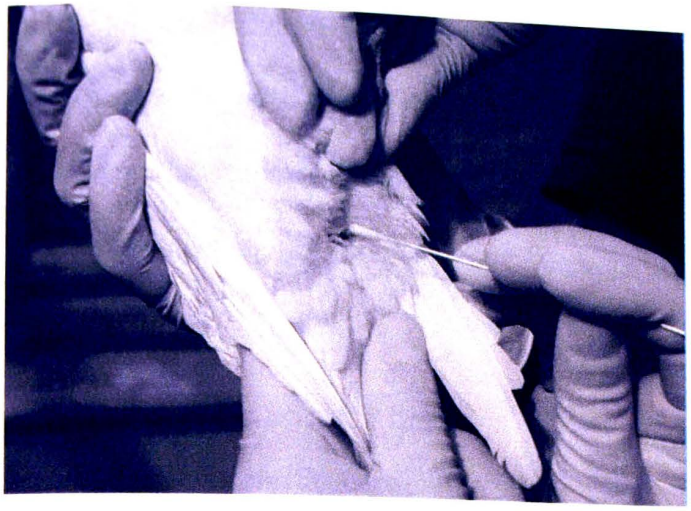

Figure 1.1: Cloacal swab procedure (Picture From LoroMania, 2013)

allows the cloacal bacteria to better adhere to a moist cotton swab compared to a dry cotton swab. Once the swab was moistened, bacteria were collected using a slight rotation of the swab for approximately five seconds around the cloaca. Once a cloacal swab had been taken, the swab was inserted into a 1.5ml microcentrifuge tube containing 500µl of sterile Difco nutrient broth, a general-purpose medium for the cultivation of microorganisms with non-exacting nutritional requirements. The cotton tip of the swab was then aseptically clipped off inside the microcentrifuge tube and the cap was closed. Microcentrifuge tubes were labeled with the date, species of bird, and the order of capture. Once the cloacal sample was taken the bird was released back into the environment.

B. Bacteria Enrichment

The cloacal swabs in nutrient broth tubes were transported back to the lab where they were placed in a 37°C incubator for approximately 24 hours to enrich the bacteria populations. Upon completion of the 24-hour enrichment period, the cotton swab was aseptically removed from the microcentrifuge tube using sterile forceps. After the swab was removed, the bacteria were collected in a pellet by microcentrifugation for five minutes. Following centrifugation, the supernatant was

poured off and the microcentrifuge tubes containing the bacterial pellet were placed in a -20°C freezer until all samples had been collected for the particular season, up to four months. Studies have shown that bacteria stored in pellets at -20°C for periods greater than ten years can still be viable after thawing (Harrison & Pelczar, 1963).

III. *Salmonella* Identification

Initially an agar based method of identification in conjunction with PCR analysis was selected for determining the presence of *Salmonella* using *Salmonella Shigella* agar. *Salmonella Shigella* agar is a moderately selective media that differentiates *Salmonella* isolates based upon their inability to ferment lactose. *Salmonella Shigella* agar also contains sodium thiosulfate and ferric citrate, which allows for the detection of hydrogen sulfide production by *Salmonella* (BD, 2006). Typically member of the genus *Salmonella* will produce colorless colonies with a black center on *Salmonella Shigella* agar, making their identification possible. After enrichment, 10µl of nutrient broth was removed from the microcentrifuge tube and dispensed on a *Salmonella Shigella* agar plate and swirled about the plate to insure the formation of isolates. Plates were then incubated for 24 hours in a 37°C incubator. After incubation the plates were removed and observed. The presence of *Salmonella* was indicated by colorless colony growth with black centers.
While this method was able to identify *Salmonella*, the numbers of *Salmonella* infections identified by the agar based method was less than the PCR results, and

was abandoned for the increased sensitivity and detection limit of a PCR based assay.

A. *Salmonella* PCR Identification

Upon completion of sample collection for the particular season, all samples were removed from the freezer and resuspended in 100µl of molecular grade PCR water. A two primer nested PCR system was used to identify *Salmonella*. Primers were selected from a previous study (Ziemer & Steadham, 2003) that revealed the 16S rDNA gene, a gene coding for a component of the 30S small subunit of the prokaryotic ribosome, (Woese, & Fox, 1977) to be a highly conserved region of DNA across all serotypes of *Salmonella* and therefore the best PCR target. Each PCR reaction was run at a 20µl volume, using 10µl Promega GoTaq® Green Master Mix, 5µl sample, 2µl primer at a 1 micromolar concentration, and 3µl molecular grade water at on a thermocycler rate of 35 cycles, at an annealing temperature of 56°C for 15 seconds, a denaturing temperature of 95°C for 30 seconds and an extension time of 45 seconds at a temperature of 72°C, hereby referred to as a standard PCR reaction. GoTaq® Green Master Mix contains *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. (Promega Corporation). Completed reactions were run on a 120ml 1.0% agarose gel and visualized using Ethidium bromide on a UV light box.

Table 1.0: Primer sequences at an annealing temperature of 56°C and a cycle rate of 35.

Primer pair target	Primer Direction	Primer sequence (5′ → 3′)
16S rDNA gene	Forward	TGTTGTGGTTAATAACCGCA
	Reverse	CACAAATCCATCTCTGGA
Nested16S rDNA gene	Forward	ATAACCGCAGCAATTGACGTTACC CGCAGA
	Reverse	GATTCTTCTGTGGATGTCAAGACC AGGTAA

VI. PCR Conditions

Prior to *Salmonella* testing, a series of controls were run using both the nested and non-nested PCR primers to ensure that the proper band size was produced and that the primers would not cross-react with other bacteria likely to be found in test samples.

A. Positive and Negative Controls

Each PCR primer pair was tested against a positive control sample of known *Salmonella* DNA, extracted from *Salmonella* bacteria using a phenol-chloroform extraction and a negative control sample containing molecular grade water in place of *Salmonella* DNA.

B. Detection Limit

The limit of detection for each primer, i.e. the smallest amount of bacteria the PCR reactions can detect, was determined using 1 to 10 serial dilutions of a live *Salmonella* culture. The dilutions were performed in 1.5ml microcentrifuge tubes containing 900µl of molecular grade water. A 100µl aliquot of the live bacterial culture was placed in tube one and vortexed to ensure that a homogenous mixture of bacteria was achieved. Next 100µl of the first tube was removed and dispensed

into another microcentrifuge tube containing 900µl of molecular grade water. This was repeated a total of 8 times to achieve a final dilution of 10^{-8}.

After all dilutions were completed, 10µl of the bacterial suspension was removed from each microcentrifuge tube and dispensed on a sterile nutrient agar plate. The solution on the nutrient agar plate was then swirled evenly about the plate to allow isolated colonies to be counted. After each plate was inoculated, they were incubated at 37°C for 24 hours and the colonies on each plate were counted.

In addition to plating, a 5µl sample was removed from each microcentrifuge tube for PCR analysis. The standard PCR reaction using the non-nested primers identified above was used to determine the limit of detection of *Salmonella* by this assay. After the non-nested primer PCR results were analyzed, 5µl from each PCR tube was removed and dispensed in a new standard PCR using nested primers. Visualization of the PCR products was achieved using gel electrophoresis on a 120ml 1.0% agarose gel using Ethidium bromide on a UV light box.

C. Cross Reactivity

Each PCR primer pair was tested against a variety of gram-negative pathogenic and non-pathogenic enteric bacteria commonly found in the intestines of warm-blooded animals to ensure that the primers wouldn't react with any of these bacteria. A 5µl aliquot of a live culture of each of the following were analyzed via our PCR assay: *Enterobacter aerogenes, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris,* and *Pseudomonas aeruginosa.* A spiked positive control reaction was included to demonstrate the ability of the assay to detect *Salmonella* in the presence

of other bacteria. Upon completion of the PCR the reaction was visualized using gel electrophoresis on a 120ml 1.0% agarose gel using Ethidium bromide on a UV light box.

V. Data Analysis

A. Statistical Tests

All statistical tests were conducted using Jmp 10 software from SAS. A contingency table, with a critical P of 0.05, was used to analyze the results of positive *Salmonella* cases for the entire season in question. The number of cases of *Salmonella* were plotted on the Y axis and season on the X. All birds tested were included in the data analysis.

A contingency table, with a critical P of 0.05, was also used to determine if there was a significant difference between families of birds. All birds captured were divided into their families, and families having only one individual were excluded from the data analysis. The number of *Salmonella* cases were plotted on the Y axis and families on the X.

The final statistical analysis using a contingency table, with a critical P of 0.05, looked at the difference between *Salmonella* infection in the two species representing the two largest families. The American Goldfinch (*Spinus tristis*) and the House Finch (*Haemorhous mexicanus*) were tested against one another for the family Fringilidae while the Tufted Titmouse (*Baeolophus bicolor*) and the Carolina Chickadee (*Poecile carolinensis*) were tested in the family Paridae.

B. History of Documented Infection in Tested Species

To determine if a species was identified as being *Salmonella* positive from our study had been previously identified as a carrier for *Salmonella*, a thorough review of the literature using JSTOR and Google Scholar was conducted searching for referenced *Salmonella* infections in tested species. Databases were searched using the species scientific name and common name as well as *Salmonella*, such as *Baeolophus bicolor*, Tufted Titmouse, *Salmonella*.

Results

I. PCR Conditions

A. Detection Limit

Colonies were labeled TNTC, too numerous to count, and individual colonies were counted.

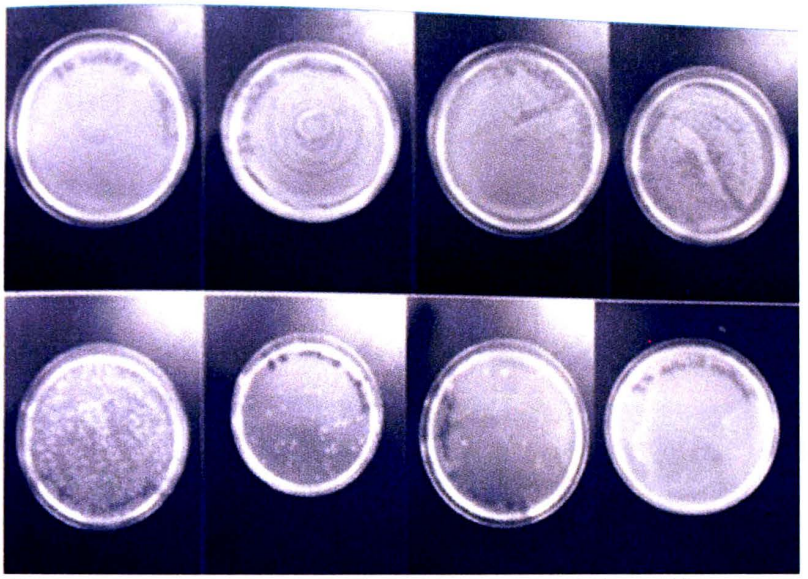

Figure 1.2: Nutrient agar plates containing *Salmonella* isolates after incubation.

Table 1.1: Amount of colonies countable on each plate after incubation.

Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8
TNTC	TNTC	TNTC	TNTC	>100	<100	9	0

After all results were analyzed a detection limit of <5 bacteria was established using the ratio of 10µl solution dispensed on a plate to 5µl dispensed in the nested PCR. Since 9 isolates were present on the 7th nutrient agar plate and the final PCR detecting *Salmonella* was the 7th, it can be concluded that half as many bacteria were present in the sample put in the PCR than were put on the plate. This results in a detection limit of <5 CFU's (colony forming units) using a nested PCR.

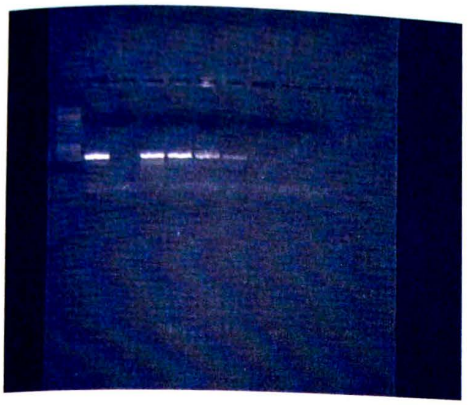

Figure 1.3: Detection limit of non-nested PCR primers.

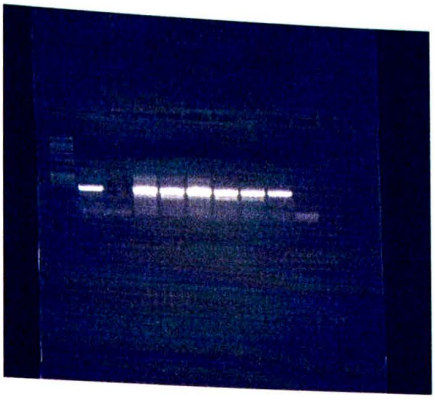

Figure 1.4: Detection limit of nested PCR primers.

B. Cross Reactivity

None of the bacteria tested against each primer showed evidence of reacting with either the nested or non-nested primer set.

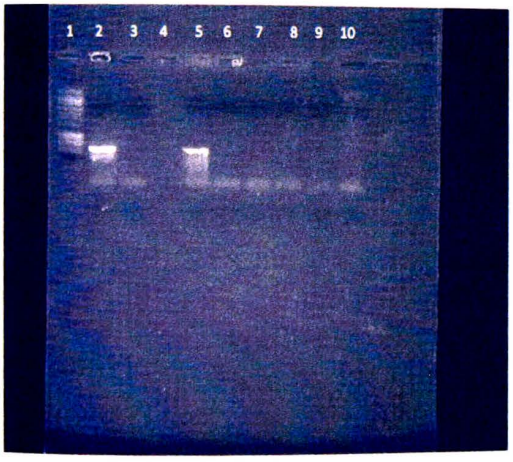

Figure 1.5: Cross reactivity of non-nested PCR primers against various gram-negative bacteria. L1: Ladder, L2: Positive control, L3: Negative control, L4: Blank, L5: Spike control, L6: *Enterobacter aerogenes* L7: *Klebsiella pneumonia*, L8: *Escherichia coli*, L9: *Proteus vulgaris*, L10: *Pseudomonas aeruginosa*.

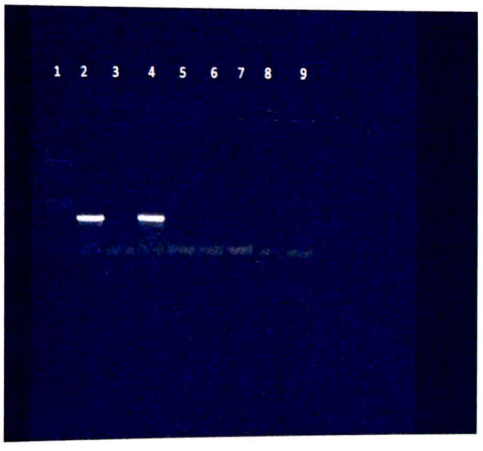

Figure 1.6: Cross reactivity of nested PCR primers against various gram-negative bacteria. L1: Ladder, L2: Positive control, L3: Negative control, L4: Spike control, L5: *Enterobacter aerogenes* L6: *Klebsiella pneumonia*, L7: *Escherichia coli*, L8: *Proteus vulgaris*, L9: *Pseudomonas aeruginosa*.

II. Trapping Results

A total of 54 birds were sampled during the summer season and 46 from the winter season, for a total of 100 birds over the entire year, representing 15 different species, 10 families, and 4 orders. Trapping took place May 22, 2013, June 21, 2013, July 7, 2013, July 23, 2013, July 29, 2013, and August 3, 2013 for the summer season while December 17, 2013, January 26, 2014, February 19, 2014, and March 14, 2014 represented the winter season.

Table 1:2 Capture data for summer.

Species	Number Tested	*Salmonella* +
Haemorhous mexicanus	21	4
Baeolophus bicolor	7	0
Spizella passerina	6	3
Spinus tristis	6	0
Cardinalis cardinalis	6	4
Poecile carolinensis	3	0
Zenaida macroura	1	1
Picoides pubescens	1	0
Archilochus colubris	1	0
Pipilo erythrophthalmus	1	0
Molothrus ater	1	1

Table 1:3 Capture data for winter.

Species	Number Tested	*Salmonella* +
Poecile carolinensis	11	2
Baeolophus bicolor	8	1
Spinus tristis	8	2
Cardinalis cardinalis	6	0
Haemorhous mexicanus	5	0
Junco hyemalis	3	0
Picoides pubescens	2	0
Melanerpes carolinus	1	1
Sitta carolinensis	1	0
Passer domesticus	1	0

III. PCR Results

A total of 13 *Salmonella* positive birds were identified using PCR in the summer season.

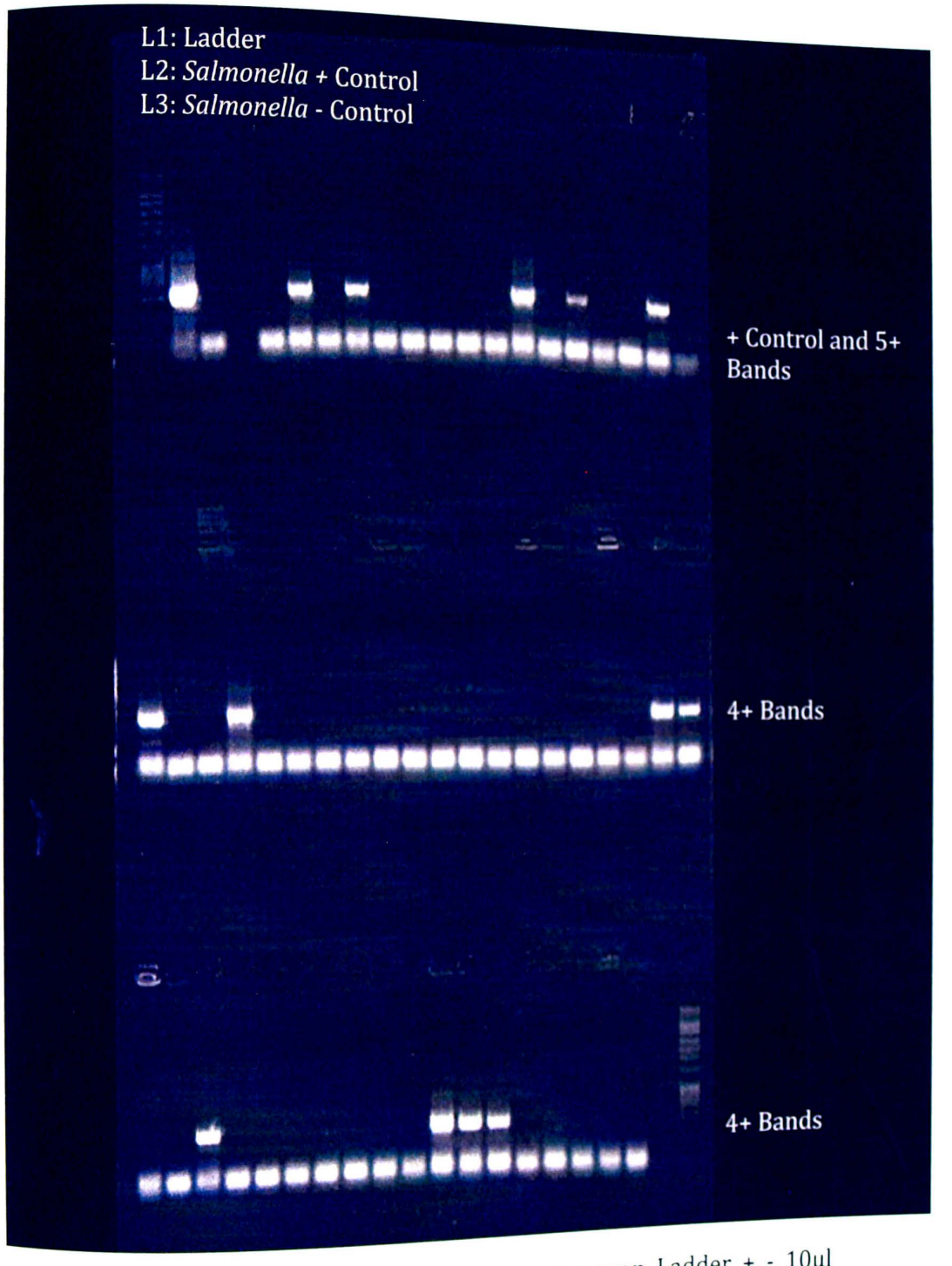

Figure 1.7: Nested PCR gel for *Salmonella* summer season. Ladder, +, -, 10µl reactions from each tested individual.

A total of 6 *Salmonella* positive birds were identified using PCR in the winter season.

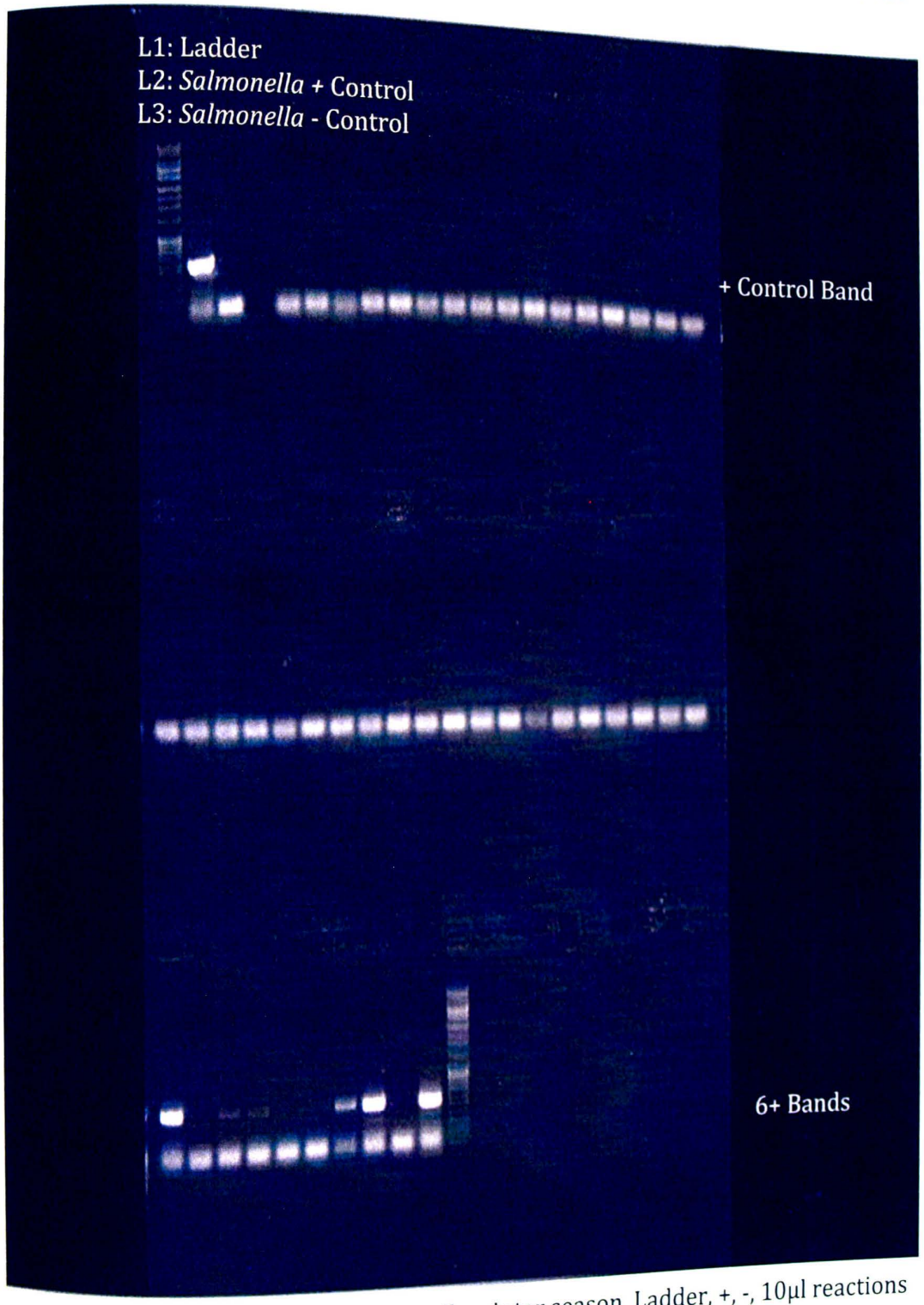

Figure 1.8: Nested PCR gel for *Salmonella* winter season. Ladder, +, -, 10µl reactions from each tested individual.

VI. Statistical Results

A. Overall *Salmonella* Infection Between Seasons

A Likelihood Ratio analyzing the frequency of *Salmonella* infection showed no significant difference between the summer and winter seasons (Likelihood Ratio: Chi-square=2.293, N=100, P =0.13) for overall *Salmonella* infection according to our data. All birds tested were included in this analysis.

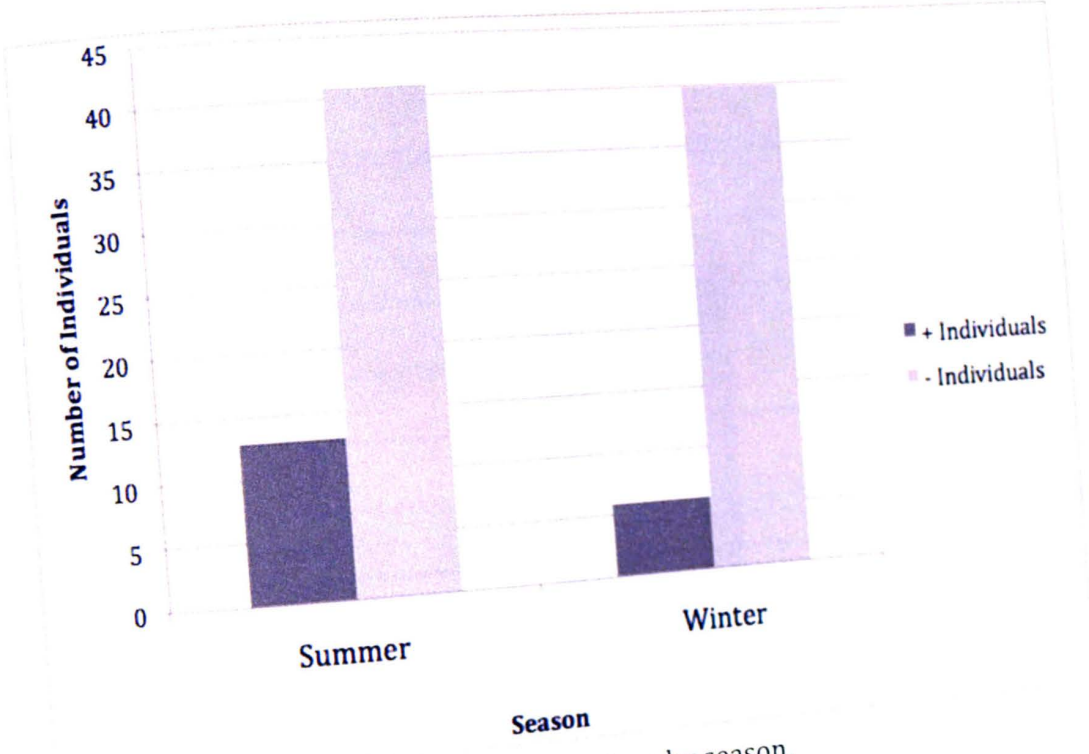

Figure 1.9: Overall *Salmonella* infections by season.

B. Overall *Salmonella* Infection Between Families

A Likelihood Ratio analyzing the frequency of *Salmonella* infection revealed no significant difference between the numbers of *Salmonella* positive individuals in any bird family (Likelihood Ratio: Chi-square=4.161, N=95, P=0.38) according to our data. All captured species were grouped according to family. Captured species were analyzed by family (families having only one representative were excluded from data analysis). The families analyzed include Cardinalidae, Emberizidae, Fringillidae, Paridae, and Picidae.

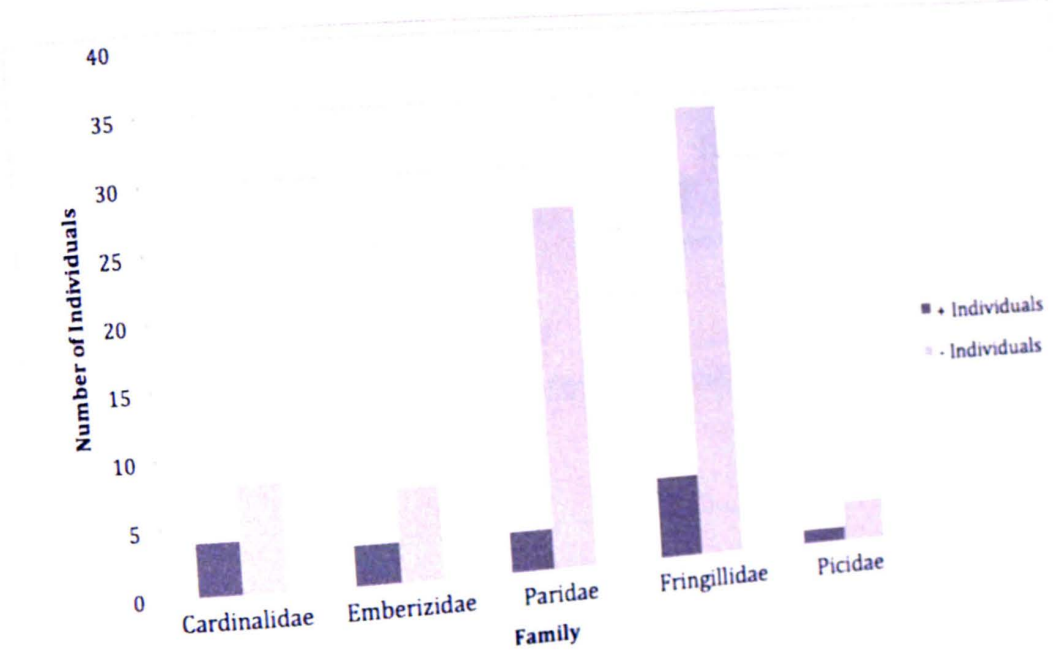

Figure 2.0: Frequency of *Salmonella* infection by family.

C. *Salmonella* Infection in the Family Fringillidae

A Likelihood Ratio analyzing the frequency of *Salmonella* infection revealed no significant difference between the numbers of *Salmonella* positive individuals for either fringillid species. (Likelihood Ratio: Chi-square=0.32, N=41, P=0.86).

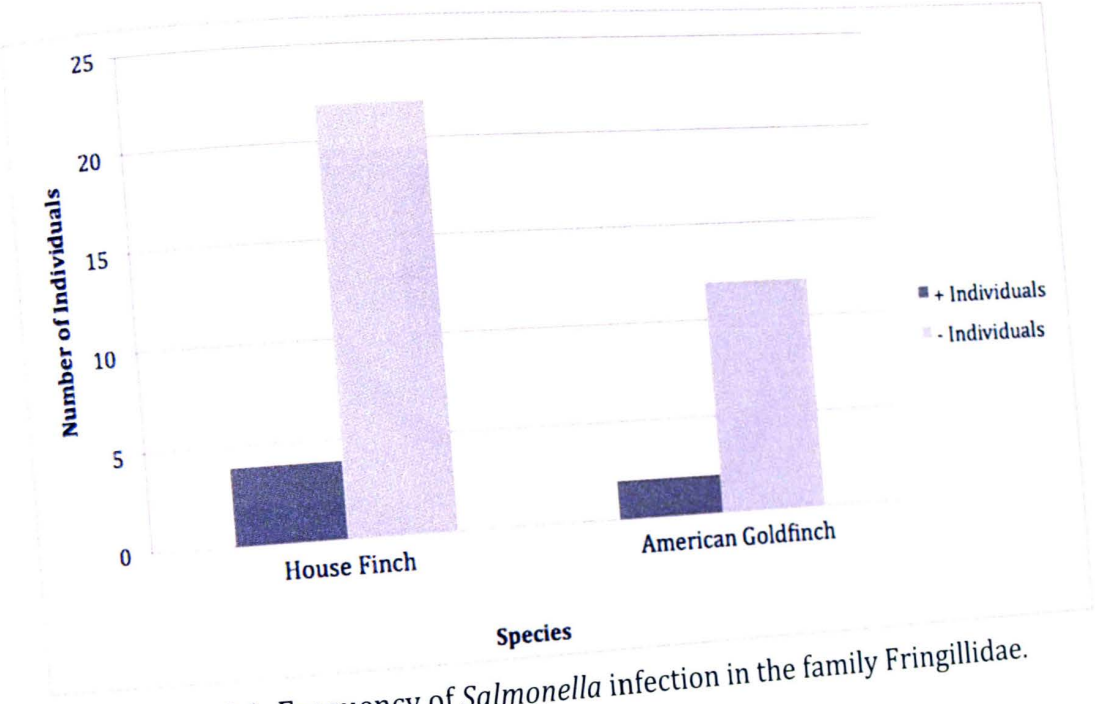

Figure 2.1: Frequency of *Salmonella* infection in the family Fringillidae.

D. *Salmonella* Infection in the Family Paridae

A Likelihood Ratio analyzing the frequency of *Salmonella* infection revealed no significant difference between the number of *Salmonella* positive individuals for either Parid species. (Likelihood Ratio: Chi-square=0.459, N=29, P value=0.49).

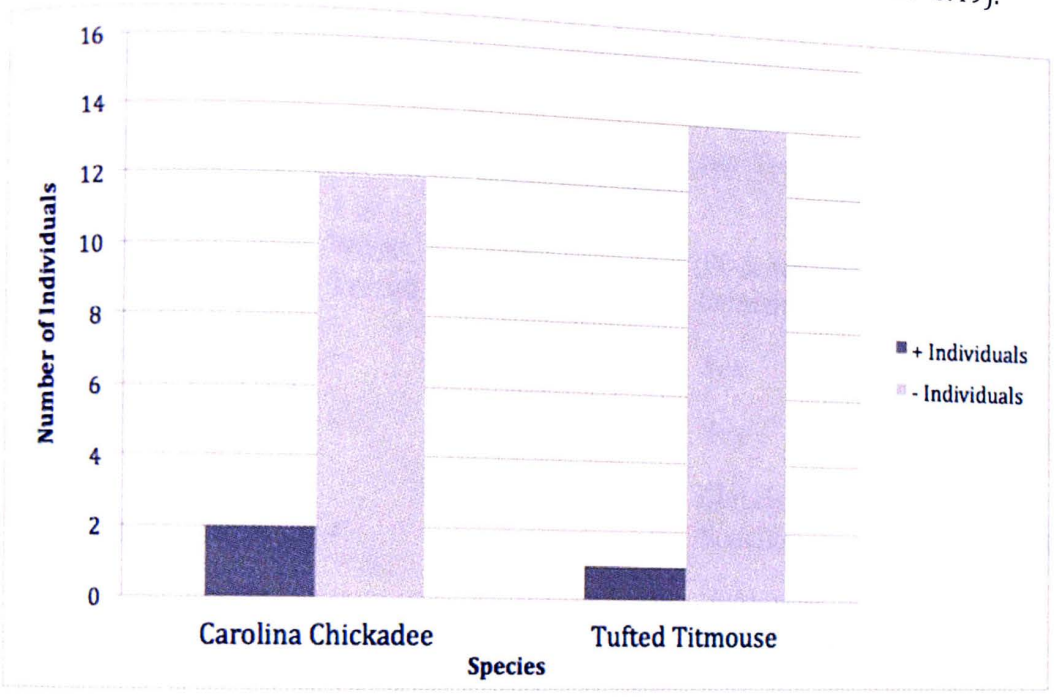

Figure 2.2: Frequency of *Salmonella* infection in the family Paridae.

V. Previously Documented *Salmonella* Infection in Species Tested

A review of the literature was conducted to determine if any other the species that were captured were previously documented as carriers of *Salmonella*. I found three previously undocumented species that were positively identified for *Salmonella* infection.

Table 1.4: Documented Infection Status on Species Tested

Species	+ or - When Tested	Sample Size	Documented Infection	Reference
Cardinalis cardinalis	+	12	Yes	Hall & Saito, 2008
Spizella passerina	+	6	Yes	Friend, 2001
Poecile carolinensis	+	14	Closely Related Species	Tizzard, 2004
Baeolophus bicolor	+	15	Unknown	
Haemorhous mexicanus	+	16	Yes	Friend, 2001
Molothrus ater	+	1	Yes	Radwin & Lampky, 1972
Spinus tristis	+	14	Yes	Hall & Saito, 2008
Zenaida macroura	+	1	Yes	Kocan & Locke,1974
Melanerpes carolinus	+	1	Unknown	
Picoides pubescens	-	4	Unknown	
Pipilo erythrophthalmus	-	1	Yes	Friend, 2001
Archilochus colubris	-	1	Unknown	
Passer domesticus	-	1	Yes	Tizzard, 2004
Junco hyemalis	-	3	Unknown	
Sitta carolinensis	-	1	Unknown	

Discussion

I found a 24% infection rate during the summer season and a 13% infection rate for the winter. This infection rate is contestant with a pervious study that found a 20%-50% infection rate for some wild bird populations (Tizzard, 2004).

While my data collected on the seasonality of *Salmonella* infection does not show a significant difference between the winter and summer seasons, previous studies suggest periods of high bird activity, such as migratory periods where large groups of birds are flocking together at feeding stations, play a role in increasing the prevalence of *Salmonella* infection in bird populations (Tizzard, 2004). Although the birds tested in this study for *Salmonella* represent a variety of species commonly found on feeders during both the summer and winter, a large number of migratory species were not captured which may have influenced my results. While some migratory birds, Dark Eyed Juncos (*Junco hyemalis*), were captured during the winter season, they represent a small percentage (6.5%) of the total birds captured (3/46 birds) during the winter season. During the periods between trapping, a large amount of migratory birds (Icteridae) were observed directly feeding on and around the feeder of at least one of the trapping locations (personal observation by homeowner). While these birds were observed at the feeder, they were not tested for *Salmonella* infection so the presence of infection in these migrants is unknown. Since all but three of the birds tested during the winter represent common non-migratory species, a base line of winter *Salmonella* infection in resident non-migratory species is best represented by this data.

Another potential factor influencing the non-significant difference between the winter and summer seasons is thought to be the amount of available food in the environment during the summer season. It is speculated that stronger, more mature birds would spend more time foraging in the environment, while weaker and juvenile birds, potentially infected with *Salmonella*, would be more frequent visitors to feeding stations. Since diseased birds commonly exhibit lethargy and are less likely to fly (Tizzard, 2004), it is speculated that they will be more common at feeders. Since the birds foraging in the environment are thought to be healthier than those commonly visiting feeders, their absence from the data could also influence the overall rate of *Salmonella* infection, although this is purely speculation.

A third factor potentially influencing the rate of *Salmonella* is the death of infected birds during the winter due to increased stress and a decreased immune system. Birds infected with *Salmonella* exhibit a decline in their health as the infection progresses. Since infected birds have a decreased immune system and a higher rate of thermoregulation they are less likely to be able to adapt to periods of increased stress. It has been documented in poultry and other domestic animals that periods of stress can lead to an increase in disease signs in infected animals (Verbrugghe, 2011) and this stress response is speculated to be the same in wild avian populations. While these birds may have been able to survive during the summer months, once the temperature began to change they were no longer able to survive and succumbed to the infection. Since these birds could not survive they were not tested for *Salmonella* and therefore represent a subset of the data that remains untested. Potentially adding these infected birds to a winter *Salmonella*

screen would cause an increase of infection rate in the winter season and represent a more accurate estimate of *Salmonella* infection for the season as a whole, instead of directly at a feeding station. While this rate of death due to a seasonal shift remains unknown it is thought to be a confounding factor.

The final factor that might have influenced the *Salmonella* infection rate of both seasons is the proximity of the trapping sites to agricultural fields and livestock. It is well known that birds frequenting agricultural fields are at an increased likelihood of contracting *Salmonella* (Gaukler *et al*, 2009). All of the chosen trapping sites are found in residential neighborhoods away from major agricultural production and livestock farms and therefore should be free of influence by *Salmonella* infections caused by agricultural impacts.

Overall, my results for the total *Salmonella* infection for both the summer and winter seasons is thought to best represent a baseline *Salmonella* infection rate for visually healthy birds during both seasons in areas free from agricultural impacts.

The lack of a significant difference in *Salmonella* infection between bird families is perhaps best explained by the small sample size representing some families and the small amount of species representing each family. In each family, two species represented the sampled individuals, the exception being Emberizidae although only one of the third species was captured. Further compounding this effect, five families were captured that were represented by a single individual. These five birds included two positive *Salmonella* infections, and were excluded from the data analysis because of statistical assumptions of 100% *Salmonella*

infection or 100% negative infection rate caused by such small sample sizes. Further testing on these particular species and a larger sample size is needed.

While the two largest families (Paridae & Fringillidae) were broken up into their representative species, a non-significant result was found in both families between infection rates of the representative species. Both the House Finch (Hall, 2008) and the American Goldfinch (Friend, 2001) are documented carriers of *Salmonella* and there appears to be no significant difference between the two species in our study. The same holds true for the family Paridae, including the Carolina Chickadee and the Tufted Titmouse. While these species are not documented carriers of *Salmonella* they both appear to have the same infection rate.

While these species represent a variety of birds commonly found on feeders they do not completely represent each species of their family found in the American southeast. Since the captured birds were the most common species observed on the feeder for both seasons during and between the trapping sessions, it is felt that these species truly represent what is commonly found on feeders year round and therefore represents a true value on the percentage of *Salmonella* infection truly found at feeders.

While an in depth literature review revealed three species of birds the Carolina Chickadee, the Tufted Titmouse, and the Red-Bellied Woodpecker that have previously not been recorded as carriers of *Salmonella*, it is likely that these birds are common carriers of infection, since *Salmonella* can infect all bird species (Friend, 2001), but have previously gone untested. In the case of the Carolina

Chickadee, infection has been documented in a closely related species, the Black Capped Chickadee (*Poecile atricapillus*; Tizzard, 2004) and therefore provides evidence that the Carolina Chickadee, is also a common carrier of *Salmonella*. Infection in the Tufted Titmouse, and the Red-Bellied Woodpecker, currently remains undocumented.

Overall this study best represents a baseline *Salmonella* infection rate for non-agriculturally impacted areas in the American southeast. Although my data shows that *Salmonella* infections are unlikely to differ between seasons, further testing should be done with a larger sample size to confirm my results.

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