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Whitney C. Pennington April 9th, 2013

A Descriptive Study of *Salmonella* in Passerines in Urban Atlanta

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Abstract

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Salmonella spp. is a genus of gram-negative, zoonotic bacterium with over 2,300 known serotypes. Though a leading cause of foodborne illness in humans, *Salmonella enterica* infection is of particular concern in birds. Field studies have found *Salmonella* background carriage rate to be as high as 8.5% in populations of passerines (songbirds) that are gregarious and live near livestock or urban areas. In these urban settings, it is widely accepted that feeding stations are associated with an increase of avian salmonellosis, specifically wintertime *Salmonella* Typhimurium outbreaks; however, it is unknown if feeders are increasing the background carriage rate of *Salmonella*. In this study we seek to quantify this background carriage rate by capturing and collecting feces from songbirds captured by mist net across four sites in urban Atlanta (Fulton and Dekalb counties). Specifically, we describe the cohort of passerines captured and outline the association between *Salmonella* carriage and other gram-negative enterobacteria and selected environmental and ornithological risk factors. We found no association between infection and sex, age, or feeding guild of bird samples. We did not find correlation between infection and the canopy cover within 500 meters of bird capture or the average number of feeders per house within each sample area. Furthermore, we found no bacterial contamination of the birds feeders located in the yards where birds were sampled. Further studies aimed at describing avian bacterial transmission in the urban environment can build on this study of 135 birds at 4 locations by selecting an alternative bacterial pathogen of focus.

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INTRODUCTION:

Avian Enterobacteria

Relatively little is known about the natural gut microbiota of birds, specifically which bacteria species make up the natural intestinal flora of different bird species. In the past, those bacteria species considered "normal" were restricted to gram-positive bacteria while any gram-negative bacteria, such as *Escherichia coli, Klebsiella pneumoniae, and Enterobacter cloacae* and *Salmonella enterica*, were all classified as acquired pathogens [1]. Because of advancements in biotechnology, mostly due to PCR, it is now much easier to examine intestinal bacterial load; however, relatively little has been explored beyond bacteria common to poultry species. Of those other avian species whose innate flora has been investigated, it is still considered unusual for gram-negative Enterobacteriaceae (such as those previously mentioned) to be cultured from Fringillidae (finch) species but not unusual for them to be cultured from other passerine (songbird) species [2]. The source of these natural bacteria remains largely unknown. In a review of current literature, Benskin et al. [3] found that acquisition of bacteria is driven by bird feeding ecology and specific environmental bird-bird contacts and that transmission risk between birds is potentially confounded by differences in behavior and physiological response to infection observed between males and females. The authors further conclude that, due to such differences, females are more susceptible to infection [3]. Similarly, given that fledging (young) birds live in the nest where they are constantly surrounded by bird excrement, exposure and immunity to gram-negative bacteria are age related, with hatch year birds being more susceptible to infection than older birds [3, 4].

Empirical evidence shows that susceptibility to enteric pathogens may not be bird species specific. A study by Morishita et al.[5] that captured wild, free living passerines concluded that

the low levels of enteric pathogen infection among their samples may have been related to the feeding behaviors and environment where the birds were collected [5]. The authors suspect that species more likely to pick through other infected sources (such as ground feeders) and those with gregarious behavior have higher probability of contacting and spreading acquired bacteria [5].

Most knowledge on bacterial infection burden in birds has originated from studies focused on zoonotic bacteria with a direct impact on human or avian health [3]. In a review of current literature, Benskin et al. [3] determined that the most commonly studied enteropathogens of birds include, among others, *Klebsiella* spp., *Enterobacter* spp., *E. coli*, *Pseudomonas aeruginosa*, and *Campylobacter jejuni* [3]. This study will focus on one such pathogen, genus *Salmonella*.

Salmonella

Salmonella enterica is a gram-negative, zoonotic bacterium with over 2,300 known serotypes [6]. Most *Salmonella* studies focus on *Salmonella enterica* subspecies *enterica* and its serotypes [7]. *Salmonella* nomenclature typically follows the following pattern: *Species genus subspecies* var Serotype. Herein we will use a "*Genus* Serotype" nomenclature unless otherwise noted as we are primarily concerned with *Salmonella enterica* subspecies *enterica*.

Broadly, *Salmonella* causes acute or chronic gastrointestinal infections in mammals and birds and is shed through fecal matter [6]. Additionally, *Salmonella* has been cultured from water sources [8-10], soil [11], food products [12], and within broiler houses [13] (Figure 1).

Salmonella is a common cause of foodborne illness and for this reason, is of great concern in the poultry industry [14]. From 1991-2012, the State of Georgia was the number one state in the country in broiler chicken production, and the state produces more than 26 million

pounds of chicken every day [15]. Because of the large presence of the poultry industry in Georgia, *Salmonella* outbreaks in humans, livestock, and wild birds are relatively common in the rural areas of the state in comparison to other states in the United States (Figure 2) [16, 17]. The Centers for Disease Control and Prevention (CDC) reports that the incidence of Salmonellosis in the state of Georgia was 27.0 and 26.6 cases per 100,000 persons in 2011 and 2012, respectively, which was well over the national averages in those years (Table 1) [18, 19]. The 2009 Salmonella Annual Summary found that the most common isolates from humans in Georgia were the serotypes Javiana, Newport, Enteritidis, Typhimurium, and Muenchen [7]. Additionally, more than ten serotypes of *Salmonella enterica* have been isolated from freshwater in South Georgia [8].

Some *Salmonella* serotypes appear to show host adaptation: they cause a specific disease in a specific host, such as *Salmonella* Typhi that explicitly causes Typhoid Fever in humans or *Salmonella* Gallinarium that causes illness exclusively in poultry [6, 12]. Yet other serotypes, such as *Salmonella* Typhimurium, are zoonotic and can infect and cause illness in many species included humans, many bird species, and cats [12]. Still other serotypes can be cultured from many species but cause active and passive infections depending on the host. For example, the previously mentioned serotypes isolated from human in Georgia (Javiana, Newport, Enteritidis, Typhimurium, and Muenchen), are pathogenic to humans, but they can persist in livestock without causing clinical illness [20].

Avian Salmonella

S. enterica infection is also of particular concern in wild birds [14]. While infection is most common in gulls and other scavenger birds, the bacterium is still present in observably

healthy songbirds (i.e. birds that show no physical signs of infection) [5, 6]. In the last twenty years, six field studies have quantified *Salmonella* background carriage rates between 0.0-8.5% in populations of passerines that are gregarious and live near livestock or urbanized sites [5, 21-25]. Those studies quantifying higher rates tended to capture birds at agricultural sites [5, 25].

Unlike the human serotypes mentioned above that do not cause clinical illness in passerine species, the specific strain *Salmonella* Typhimurium is considered a significant source of avian mortality and is transmitted through the contaminated environment although egg transmission can occur [14, 26]. In urban passerines, outbreaks show a highly seasonal pattern, occurring in the winter months from December-March caused nearly exclusively by the Typhimurium serotype [6, 26]. Bird feeders are often implicated as the source of infection of these outbreaks because these artificial feedings stations increase fecal-oral contact and social stress related to large colony gatherings and social interactions [27, 28].

Bird Feeders and Bird Health

The role of bird feeders in altering avian ecology and behavior has been highly scrutinized. Concerns about the impacts of feeding include enhancement of introduced species, bird dependence on human-provided foods, nutritional deficiencies of supplemented food, loss of foraging skills, changes in migration, increased aggression, and spread of disease [29]. A review of birds deaths submitted to the National Wildlife Health Center (NWHC) suggests that over 50% of events leading to bird death were associated with feeders [26]. Diseases commonly referred to as feeder diseases and thought to have feeder-associated transmission include salmonellosis, trichomoniasis, aspergillosis, avian pox, and mycoplasmal conjunctivitis [29, 30]. Salmonellosis was determined as the primary or secondary cause of death for 5% of events

reported to the NWHC, representing more than 68,000 birds recorded between 1985 and 2004 [26]. In one study, the number of bird deaths found at feeders have been positively associated with bird density and species diversity of birds using the feeders; however, it is impossible to know if disease was truly the cause of death in these cases since necropsies were not performed [31].

While massive die-offs have been documented around feeders, the lack of microbiological confirmation of *Salmonella* spp. or other pathogens infecting the surface of these feeders precludes any direct association between feeder presence and bird mortality [27]. Other authors have used a lack of birdfeeder exposure to justify low bacterial prevalence in passerine species; however, relatively little is known about the exact dynamics of disease ecology around bird feeders [21].

There is concern that a trend towards year-round feeding in urban areas where bird feeder predominate is increasing the background carriage rate of *S. enterica* in backyard birds overall [32]. However, it is not known how these feeding practices may be altering exposure, and thus susceptibility, of passerines to the common outbreak strain. Additionally, as areas of frequent congregation, feeders could potentially increase the risk of non-outbreak, primarily livestock-adapted, strains being introduced and transmitted in urban areas.

While the exact mechanisms remain unknown, zoonotic transmission of bacteria has been shown to occur as the result of predation on wild birds by cats [33]. Backyards may play a role in this zoonotic transmission pathway as they are a common environment and gathering point for humans as well as avian and other nonavian susceptible populations (such as cats or dogs). Moreover, playing in an area with bird droppings, cleaning or removing bird droppings, and direct contact with birds or bird droppings have all been found as significant risk factors for

human Salmonellosis and suggest that bird feeders are of public health concern [34] Collecting, analyzing, and interpreting data on background carriage rate may shed light on the impact of bird feeders on bacterial carriage and also the need for education surrounding proper management of residential bird feeders.

Objectives and Hypotheses

Given that *Salmonella* infection is prevalent is the state of Georgia as previously discussed, we hypothesize that there is spillover into the urban environment of Atlanta. The objective of this study is to quantify the carriage rate of *Salmonella* in an urban population of presumably healthy songbirds that may supplement their diet at bird feeders. We captured and fecal-sampled wild free-living passerines from four areas across Atlanta, Georgia to describe difference in the prevalence of *Salmonella* and similar gram-negative enteric bacteria. We examined the relationship between the proportion of infected birds and canopy cover, bird feeder density, and avian species richness across sampling locations to determine aspects of the urban environment that may be associated with transmission. Additionally, we sampled the bird feeders in the vicinity of capture sites and attempted to culture the same bacteria.

For the purposes of this study, we chose to follow the avian bacteriology paradigm that passerine species are not naturally infected with gram-negative bacteria thus any such infections are environmentally acquired not innate [2]. We acknowledge that this may not be true of every individual bird, as Benskin et al. [35] have shown that innate bacterial infection among captive raised passerines is highly diverse [35]. However, we believe the sensitivity of our methodology reflects infection in high concentrations indicative of repeated environmental exposure and

acquisition beyond innate infection. Moreover, we will refer to this carriage as infection but acknowledge that these bacteria are not causing observable clinical illness.

We hypothesized that greater density of bird feeders in a given urban space will present increased opportunities for birds to supplement their diet at a feeder and thus potentially contact *Salmonella* or other enteric bacteria transmitted on or around feeding stations. Therefore, we predict that the prevalence of *Salmonella* in passerines would be higher in areas with higher feeder density. Similarly, the prevalence of gram-negative enteric bacteria would also be higher in areas of higher feeder density. Conversely we predict that bird populations living within more forested areas would have less contact with feeding stations and therefore a lower prevalence of *Salmonella* and similar gram-negative enteric bacteria. Thus, we hypothesized that prevalence of infection would decrease as percent canopy cover increased.

MATERIALS AND METHODS

Study Sites

From June-October 2012, birds were live-captured using mist-nets at four areas across the metro Atlanta area in Fulton and DeKalb counties of Georgia, USA (Figure 3). These areas were chosen because they are the sites of ongoing research of West Nile Virus in birds. Briefly, these areas are predominantly residential, but include commercial areas, and are in close proximity to large public parks. Areas in Fulton County consisted of groupings of two to four residential yards, called sites, where at least one of these yards contained a feeder maintained throughout the sampling period by the homeowner (Figure 4A-C). Additional nets were set in adjacent yards to increase the probability of collecting birds interacting with a local feeding station. Homeowners were identified through previous studies on avian diseases in this area (Rebecca Levine, personal communication). A single area in DeKalb County on the property of Emory University within Lullwater Preserve served as a control area without any known bird feeder use within a 500m radius. Within Lullwater Preserve, nets were set inside and directly adjacent to the gated research pond in the forested eastern portion of the preserve (Figure 4D).

Mist Netting

One to two mornings a week, four to seven 6-m and 12-m nylon mesh mist nets were used to capture live birds (Avinet, Inc, New York, NY) (Figure 5A and B). Sampling was conducted on fair weather days between the hours of 6:00am and 12:00pm. Nets were checked every 40 minutes, and all captured birds were removed and individually placed in medium-weight brown paper bags for no more than 30 minutes. After defecation in the bag, each bird was banded, identified to species, sex, and age (where possible), measured for mass and wing length, and released in the same area. Before release, feces were collected from the paper bag using a

sterilized toothpick and/or the bird's cloaca was swabbed using a calcium-alginate urethral swab wetted with sterile saline (Figure 5C and D). All sampling was done in accordance with Emory Institutional Animal Care and Use Committee (permit# DAR 2001632-050815), Georgia Department of Natural Resources (permit# 23722), and the United States Geological Survey Bird Banding Laboratory (permit# 23673). Collected feces and swabs were placed in 2ml cryovials and stored on ice and transported on ice to the laboratory. All samples were stored at -80°C within 5h of collection.

Salmonella Cultivation

Samples were removed from the freezer and allowed to come to room temperature before being added to 3mL sterile Buffered Peptone Water (BPW) and incubated for 18-24h at 41.5°C. Fecal samples were preferentially selected for cultivation over cloacal swabs where both existed. In instances where no feces were collected, swabs were used. Approximately 0.5mL of the BPW solution was added to 4.5mL sterile Rappaport-Vassiliadis Enrichment Broth (RVB) and incubated for 18-24h at 41.5°C. Each RVB sample was then streaked onto two *Salmonella* selective Xylose Lysine Tergitol-4 (XLT4) agar plates using sterilized cotton swabs and incubated at 41.5°C. Plates were checked at 24 and 48h after streaking, and presumptive colonies were restreaked on XLT4 plates for purification. All media were prepared according to manufacturer's instructions. Additionally, a negative control sample was included in each step of cultivation to ensure against contamination of all media.

Feeder Testing

In February 2013, the surfaces of feeders within the yards where birds were captured were sampled. Upon entry to these yards, feeders were observed for five minutes before being approached. Two sterile cotton swabs were then rolled across the feeder, each one covering all potential perching surfaces of each feeder. The length of swabbed surface was then measured using a measuring tape. Swabs were placed in individual sterile whirl-pak bags and kept at air temperature until processing (less than 4h). Additionally feeder type, seed type, and approximate volume of seed remaining were recorded for each feeder. On the same day, whirl-pak bags were filled with 10mL of prepared sterile BPW and shaken vigorously. Then 2mL of this solution was removed and incubated for 18-24h at 41.5°C. Cultivation then continued as described above for fecal samples and swabs.

Standardization

Due to differences in weather and availability of sites for sampling, the total time spent mist netting at each site was not equal. To make comparisons between the samples caught at each site, we standardized samples by calculating a catch per unit effort (CPUE) at each site. First, we calculated the cumulative effort for each site as meter-hours: the total length of net open multiplied by the hours it was open for each sampling day. To calculate CPUE for each site, the cumulative number of samples collected at each sampling period was divided by the calculated cumulative effort. Effort and CPUE were calculated in a cumulative fashion because the parameters of interest were the overall effort and CPUE for each area, as these were the values that would be compared in analysis.

Area Specific Measures

The locations of each sampling site were geocoded into ArcGIS (ESRI, Redlands, CA) and around each sampling site a circular buffer with radius of 500m was created. This buffer size was chosen based on home range and population spacing of Northern Cardinals, *Cardinalis cardinalis* (the most common bird in Atlanta backyards) as described by the Cornell Ornithology Lab [36]. Using maps of land cover in Fulton and DeKalb counties, each buffer was then divided into 30m by 30m pixels. Each pixel was classified as vegetated or not vegetated. The percent canopy cover for each buffer was calculated as the proportion of pixels classified as vegetated. Each study area's percent canopy cover is the average of its site's percentages.

Feeder density at a given area was calculated as the average number of feeders per house at each site in the area. For example, we sampled from three sites in the Grant Park North area. Two houses had three feeders each and the third had one feeder. Thus site specific feeder densities were 3, 3 and 1 respectively. Feeder density is then 2.33 feeders per house as it is the average of these three values. This value does not represent the feeder density within an entire buffer, but is an estimate of the feeder density in the portion of the buffer where birds were captured.

Individual Specific Measures

In addition to the measurements made in the field, after collection, each species was assigned to a feeding behavior group based on behavior data from the Cornell Lab of Ornithology's Bird Guide [37]. Specifically, each species was classified as: bark forager, fly catcher, foliage gleaner, or ground forager. This classification was made independently of diet, so those considered ground feeders could be both seed eaters and insectivores.

Statistical Methods

Infection status was separately tested for independence from age, sex, and feeding guild using a Chi-squared test or a Fisher's Exact test if sample size was small (less than 5). Additionally, correlation analysis was used to check for associations between infection and each landscape variable. All statistical analyses were run in SAS version 9.3.

RESULTS

Landscape

Both canopy cover and feeder density (measured as average number of feeders per house) differed across the four sampling areas (Figure 6). Not surprisingly, Lullwater Preserve, a forested, natural area within an urban environment, had the greatest percent canopy cover (50.9%) as well as the smallest feeder density (0 feeders/house). Grant Park East had a similar percent canopy cover (40.9%), while the other two areas, Piedmont and Grant Park North has markedly lower percent canopy covers (25.0% and 15.3% respectively). Grant Park North, the area with smallest percent canopy cover, also had the greatest feeder density (2.3 feeders/house). The other two areas, Piedmont and Grant Park East each had intermediate feeder densities, 0.5 feeders/house and 0.7 feeders/house respectively.

Bird Samples

Throughout the entire sampling period, nets were open for more than 50 hours. Because of differences in availability of sites and weather conditions on sampling days, net operation was not constant at each area and thus the sampling effort (meters*hours) were not equivalent across all sites (Figure 7A). Specifically, extreme heat in the middle of our sampling period impacted net operating hours, and one scheduled visit to Grant Park East was cancelled due to a thunderstorm. Additionally, in Lullwater Preserve, dense foliage severely limited our ability to set nets, and as a result we were only able to use one 12-meter net in that area.

In total, 140 bird captures were made from which 135 samples were collected across all areas (Table 3). In this study, a sample is represented by 1-3 cryovials containing feces and/or a cloacal swab. These samples represent 19 species of passerines and woodpeckers (Table 4).

Overall, the most frequently sampled species was the Northern Cardinal *Cardinalis cardinalis* (n=51, 37.8%), even though it was only captured in three of the four areas. Only two species were captured at all four areas, Brown Thrasher *Toxostoma rufum* and Carolina Wren *Thryothorus ludovicianus*. The majority of samples (n=82, 60.7%) as well the most diverse number of species (16) were captured at the Piedmont site (Table 3). Additionally, six species were unique to the Piedmont area (American Goldfinch *Spinus tristis*, Brown-headed Cowbird *Molothrus ater*, Downy Woodpecker *Picoides pubescens*, Eastern Wood Pewee *Contopus virens*, Northern Waterthursh *Parkesia noveboracensis*, and Song Sparrow *Melospiza melodia*), three were unique to Grant Park North (Blue Jay *Cyanocitta cristata*, Mourning Dove *Zenaida macroura*, Tufted Titmouse *Baeolophus bicolor*), and no unique species were captured in either the Lullwater or Grant Park East areas (Table 4). Seven birds were recaptured and sampled twice throughout the sampling period: one Song Sparrow, one Carolina Wren, and five Northern Cardinals. All recaptures occurred at the Piedmont area. With the exception of two House Finches *Carpodacus mexicanus*, with symptoms of mycoplasmal conjunctivitis (swollen, watery, or crusty eyes), all birds appeared observably healthy.

The catch per unit effort (CPUE=N samples/sampling effort per area) differed between areas (Figure 7B). CPUE was much greater in the Piedmont and Grant Park North areas than the Lullwater and Grant Park North areas. This difference in CPUE appears to coincide with differences in percent canopy cover, with the two lesser forested areas (Piedmont and Grant Park North) having CPUE's 7-9 times greater than the forested, Lullwater and Grant Park East areas.

Among birds that could be aged, the majority (n=86, 67.7%) were adult birds, identified as being after-hatch year based on breeding status or plumage features (Table 3). Since capture occurred during the mid or late summer when many species were not actively in breeding season

and had already molted out of their juvenile plumage, sexing some birds was difficult. Of the 135 samples collected, only 80 (59%) could be reliably sexed. Of these, sex ratio was fairly equal with 45 females representing 56.3% and 35 males 43.8%.

The majority of birds captured were classified as "ground foragers" (n=128, 94.8%). Additionally, five birds (3.7%) were classified as "foliage gleaners" and only one bird was classified as each "bark forager" (Downy Woodpecker) and "fly catcher" (Eastern Wood Pewee) (Table 4).

Feeder Sampling

In total, 11 artificial feeding stations were sampled. Two were sampled in the Piedmont area, a tube feeder and a hanging platform feeder filled with water. Both were from the same house and none of the three additional houses in that area contained maintained feeders. Two stations were sampled from the Grant Park East area. Both were tube feeders, but each was located at a different house. The third house in this area did not maintain a feeder. The Grant Park North area had the most stations. One house maintained a single platform feeder. The two other sites maintained three feeders each. The second house had a tube feeder, suet feeder, and platform feeder. The third house maintained one suet feeder and two tube feeders. At houses where multiple feeders were maintained, stations were grouped together, and located in the same portion of the yard.

Plate Culture

Of the 135 samples tested, none showed growth of *Salmonella* spp. While *Salmonella* is the primary bacteria expected to grow on the specific culture medium, XLT4 agar, several other gram-negative bacteria were able to grow due to the enrichment process. These bacteria include

Enterococcus, E. coli, Proteus, and *Citrobacter* [38]. Due to limited sensitivity of the methods, these species could not be differentiated by plate culture alone. For the purposes of this study, we did not conduct any further testing to differentiate between these species.

Overall, 91 samples (67.4%) exhibited growth of at least one type of gram negative bacteria (Table 5). Typically this growth consisted of dense lawns made up of yellow and pink colonies (Figure 8). Although we will use the term infected to describe the presence of these bacteria, because we did not identify them to species, we do not know whether their presence is due to active infection or passive carriage. The samples collected at the Lullwater and Grant Park East sites had a lower prevalence of gram-negative bacteria (50% each) while the Grant Park North area had a higher prevalence (80.5%) (Table 5). There was no relationship between site and infection (Fisher's Exact=3.09, p=0.14). Additionally, none of the 11 feeder samples showed growth of *Salmonella* or any other gram negative bacteria (Table 5).

Infection and Landscape

In general, infection appears to be negatively correlated with canopy cover (r=-0.96, p>0.06) and positively correlated with feeder density (r=0.90, p>0.10) and species richness (r=0.77, p>0.23), however these correlations were not significant, possibility due to our limited sample size (4 sites) (figure 9A-C). Within all of these associations, the Grant Park North site appears to have the strongest influence because of its high 80.5% prevalence of gram-negative infection.

Infection and Individuals

When samples from birds of unknown age were excluded, there was no association between age and infection (χ^2=0.40, p>0.53) (Figure 8D). Similarly, when samples from birds of unknown sex were excluded, there was no association between sex and infection (χ^2=0.73, p>0.39) (Figure 8E). Because the range of feeding behaviors was limited and almost exclusively consisted of "ground feeders", we did not test for an association between feeding behavior and infection (Figure 8E). This biased distribution is most likely the result of most net location. Nets stand about 2.5 meters tall, and with their proximity to feeders, were more likely to capture birds that are active in the lower canopies and near the ground.

DISCUSSION

Salmonella Infection

Studies assessing the intestinal bacteria of avian species have similarly found no *Salmonella* prevalence in free-living passerine and near passerine species (i.e. Woodpeckers) [24, 25, 39]. While the prevalence found in this study is consistent with some literature values, it may also be driven in part by the limited sample size. Not isolating *Salmonella* from any of the samples does not necessarily mean that its true prevalence is 0% but rather that we failed to capture any in such a limited sample. With conservative estimates of prevalence at or near 1%, the expected result from our 135 samples was only 1 positive sample. Alternatively, *Salmonella* may simply not be an important part of the bacterial community in urban Atlanta.

While this 0% prevalence is not surprising overall, it is interesting in the context of *Salmonella* transmission in Georgia. The bacteria has been isolated from free-living birds and water sources in the region, although these areas are typically less urban and in closer proximity to agricultural sites, as shown by other studies [8, 23]. Despite its presence in the region, it is possible that the bacteria is not as prevalent in urban centers because there is no significant interaction between urban and rural birds and sites. In the future, isolation of livestock adapted, non-Tyhpimurium strains in these urbanized sites may suggest interactions are occurring with agricultural areas.

Furthermore, this study focused specifically on local birds (with the exception of 2 Northern Waterthush, all birds species are native to the area year-round) that would not be expected to have agriculture exposures. In these primarily urban landscapes, one might expect to isolate a Typhimurium sample as it is the serotype that most commonly infects passerine species

[14]. Prevalence of 0% in the summer months suggests that its transmission may be limited to the winter season when increased stress makes birds more susceptible to infection [6].

Additionally, it has been shown that gulls and shore birds show higher prevalence of *Salmonella* because they more often live and eat in sewage outfalls whereas urban passerines do not have similar exposure [3, 40]. In urban environments, human waste remains almost exclusively subsurface, limiting the contact that passerines may have with any infected human materials and thus eliminating human waste as a source of infection for these birds. The exception to this is surface level waters that experience sewage influent as the results of combined sewer overflow (CSO) events. However, a study by Plant [40] found that the prevalence of *Salmonella* in passerines actively feeding at an open sewage plant in England was still less that 1% [40]. Plant [40] only isolated *Salmonella* from a single captured bird, and furthermore the specific serotype isolated was not consistent with those isolated from the sludge itself [40]. This suggests that sewage products may not be as important in passerine infection as in gull infection but sufficient research has not been able to confirm this hypothesis.

Gram-Negative Infection and Individual Specific Measures

Having isolated no *Salmonella*, it is impossible to determine the relationships between infection and the landscape and individual variables we measured; however, we did find that 67.4% of samples exhibited bacterial growth of some kind (Table 5). On an individual level, this growth was independent of age and sex, when determinable. This study does not confirm that hatch year birds or female birds are more susceptible to bacterial acquisition, as literature suggests [3].

Since the majority of our samples came from ground foraging birds, we could not determine if infection was associated with feeding behavior. It is interesting however that bacteria was cultivated from the samples of both the bark foraging and fly catching species (Downy Woodpecker and Eastern Wood Pewee, respectively) as they are less likely to contact a bird feeder. While it is difficult to draw any substantial conclusions from just two birds, their infection suggests there are other environmental reservoirs mediating infection besides bird feeders or that these bacteria may be part of the natural intestinal flora. These infections might also occur very early in life in the nest environment or be transmitted by a bird's parents. Since little is known about the autochthonous bacterial species of the bird gut, future studies to better define these species will enhance our ability to interpret the relationships between infection and ornithological risk factors.

Gram-Negative Infection and Area Specific Measures

Of the landscape variables, percentage canopy cover in the 500 meters surrounding the location of bird capture appeared to correlate most strongly with infection. As hypothesized, the prevalence of infection decreased as canopy cover increased (Figure 9A); however, the strength of this correlation is based on only four different areas and is severely limited by this small sample size at both the Lullwater and Grant Park East areas. When looking at the two areas where we have the most information on infection, Piedmont and Grant Park North, we see what could become a decreasing pattern of infection with increasing percent canopy cover, but with only two areas to compare, we cannot comment on overall significance.

Associations between canopy cover and infection are also limited by the uncertainty of bird home range. The defined 500 meter area is an estimate that does not accurately reflect the

exact home rage of each individual bird; the 500 meters could both underestimate or overestimate the actual range. It is also possible that original infection did not occur within the range and thus canopy cover has no correlation with infection at all.

Interestingly, the less forested sites had CPUE's 7-9 times greater those more forested sties, resulting in the capture of fewer birds representing few species from areas with greater percent canopy cover. In these more forested areas Carolina Wrens made up 50.0% of the total samples while they made up less than 10% of samples in the other sites (Table 4). This suggests that community of passerines in general and in netting locations may be different in forested areas as opposed to less forested areas. It is also possible that the correlation we observed between canopy cover and infection is driven by a third species specific infection pattern more easily seen in an analysis of community structure. In this study, we cannot assess the impact of community structure on infection as we did not estimate any parameters of community structure beyond species richness.

We estimated species richness based on the number of species from which we collected samples. This estimate is biased as species richness is likely a function of how many birds were captured at a specific location since areas where more birds were captured had greater species richness. Of our four sampling areas, the values of species richness for the Grant Park North and Piedmont areas are less biased estimates of true species richness of birds visiting feeders in those areas than the Lullwater and Grant Park East values of sample species richness because of differences in area specific sample size (Table 3). We could overcome this bias by conducting community level population analysis; however, we are interested in the diversity of species at feeder locations, so using richness of samples may be a better estimate than overall community richness.

Surface Contamination of Bird Feeders

Surveillance of the bird feeders in these areas did not reflect the same bacterial prevalence as the birds. It was hypothesized that for feeders to play an important role in the transmission of these bacteria, the bacteria would also be present on feeder surfaces. We did not cultivate any bacteria, *Salmonella* or other gram-negatives, from the surfaces of 11 feeders. The lack of *Salmonella* was expected after no *Salmonella* was cultivated in bird samples; however we did expect to cultivate other gram-negative bacteria. The most likely explanation for this is not an overall absence of bacteria but insensitive cultivation methods. Bacteria collected from environmental samples is less concentrated and less viable (because of exposure to the environment that may cause damage) than that found in feces. In this state, these bacteria are more easily inhibited by the cultivation method. Therefore the results from the feeder sampling are more accurate in the sense that they represent true *Salmonella* negatives; however they cannot be used to judge the overall hygiene of the feeders. Interestingly, we did capture two House Finches that had symptoms consistent with mycoplasmal conjunctivitis, Avian Eye Disease. Laboratory experiments have shown that indirect transmission of the causative pathogen, *Mycoplasma gallisepticum*, can occur in a bird-bird feeder-bird cycle [41].

Limitations

One limitation of this study, and similar assessments of gut microbiota, was the cultivation method, as there is no gold standard method for the detection of *Salmonella*. Agar plate cultivation of *Salmonella* requires both pre-enrichment and enrichment steps as well as plating on selective media because the bacteria is generally present in smaller concentration than other bacteria and must be amplified to be cultured. Our protocol followed those of similar studies [5, 22-25], nevertheless, the literature does cite alternatives that may increase cultivation

sensitivity. This includes alterations in pre-enrichment and enrichment that increase sensitivity, shorten cultivation time, and increase purity with which *Salmonella* isolates can be grown [42-46]. However, most of these methods are specific to samples taken for poultry or swine products or to cultivation of serotypes adapted to these species [42, 43, 45].

Regardless of the exact methods used in plate cultivation, false negative results due to overgrowth of other bacteria are a common concern. Other gram-negative and gram-positive bacteria present in samples, pathogenic or otherwise, can prevent growth of *Salmonella* if they are present in high densities. This is especially true of samples cultured from feces because of their high bacterial load. Of the 67% of our plates where bacteria outgrew the inhibitory powers of the cultivation method, a large portion of these plates grew dense lawns that completely covered the plate and could have overgrown potential *Salmonella* colonies. The use of PCR methods is typically more sensitive in these instances as it is generally more sensitive to lower bacterial concentrations, which is often the case for *Salmonella* [3]. However, due to homologies of *Salmonella* and other gram-negative bacteria, the test may be oversensitive and confirm false positives (non-salmonella bacteria as salmonella) [47]. Increasing the sensitivity to *Salmonella* may require primers specific to individual serotype [48]. With more than 2,300 serotypes known, increased sensitivity requires added complexity.

Ultimately, cultivation method is determined by the investigator and is driven by access to resources. In this particular study, agar plate cultivation, while perhaps less sensitive and more time consuming, was chosen because it is the method used in much of the literature [3, 5, 22-25]. Creating a protocol for plate cultivation of 135 samples was easier to compile, as creating PCR protocol would have required time and resources that were not available. In the future, PCR may

be the preferred method in two instances: if a larger sample size is collected and if samples will be screened for additional bacteria.

Conclusion

Our study confirms the rarity of *Salmonella* infection within the urban environment. Moreover, with such limited data collected, the findings of this study, a significant correlation between canopy cover and infection does not have enough weight to suggest these phenomena are occurring. This study cannot confirm any causal relationship between canopy cover and bacterial infection, however it gives cause to investigate further the impact of landscape variables on avian bacterial infection and their possible associations.

Future Directions

To increase sensitivity of bacterial cultivation, in the future we are strongly considering the use of PCR. This change of methods will also allow us to screen for many more bacteria and potentially attempt to define the suite of bacteria that are present in the gut of urban Atlanta's songbird populations. Since this type of study will be much more cultivation intensive, its focus will be on a finer scale: one neighborhood or one particular species. Whether or not we choose to expand to PCR techniques, any continuation of the current study will require devising methods for indentifying those species that grow in conjunction with *Salmonella* and/or another, more ubiquitous bacteria that may serve as an indicator species.

This study would benefit from an increase in sample size, both of birds and sampling areas. While increasing sampling days is one obvious way to increase sample size, expanding the number of nets set at each area may increase bird captures more efficiently than adding sampling days. Moreover, a simple change in data collection could increase the number of areas. In this

study we grouped nets into areas, recording the area, not individual net where a bird was captured. By recording net capture as opposed to area capture, we will automatically increase our number of sampling areas. However, since bird home range is generally larger than one yard, the independence of sites within an area should be considered before an area is broken up. Since this study determined a possible association between canopy cover and infection, future sampling sites may be recruited based on their canopy cover such that sites represent a continuum of canopy cover percentages relevant to urban communities. When sampling at these new areas, all efforts should be made to ensure that sample collection is fairly consistent between areas such that location level infection is not skewed by a small sample size by increasing sampling effort, if possible.

In the future, much can be done to address the dearth of information regarding the role of feeders in bacterial transmission in the urban environment. To improve feeder sampling, methods need to be improved to increase sensitivity. This includes expansion of bacterial screening such that comparisons may be made based on bacterial density and not just presence or absence. The ability to collect more in depth information about feeder contamination, both qualitative and presence/absence, allows for exploration of spatial and temporal patterns in bacterial density. If these studies reveal differentiation in feeder contamination, then it may also be necessary to investigate the impact of feeder type on bacterial density. We are currently expanding this study to include data on the use of feeders and practices of feeder hygiene where birds were captured. These data, paired with data on the surface contamination of these feeders, may shed light on associations between feeding practices and bacterial presence and abundance. Through this survey process we will also obtain an estimate of the total number of feeders within our 500m buffers and can calculate a feeder density representative of the entire home range of the samples

we collect. Further investigation of feeders may also incorporate semi-natural experiments where homeowners are prescribed hygiene practices and timelines, the impacts of which are assessed on the bacterial communities of feeder surfaces and local birds.

Using the data from this study, we preformed a power analysis to estimate the sample sizes needed to detect significant results (at 95% power) for gram-negative infection by age and sex. Given that we captured twice as many adult birds than juvenile birds, we estimate that we would have to sample a minimum of 86 adults and 43 juveniles to detect a significant relationship between age and infection. This estimate of 129 samples is slightly larger than the 127 samples collected in the current study, and overall capture would have to exceed 129 to make up for the portion of birds whose age cannot be determined. Those numbers will be largely increased if comparisons across study sites need to be made. Our power analysis suggests that further exploration of ornithological risk factors and bacterial infection in urban birds would best be done using a bacteria species with a greater effect size. However, a significant relationship between age and infection may be determined without much increase in sampling effort.

Figures

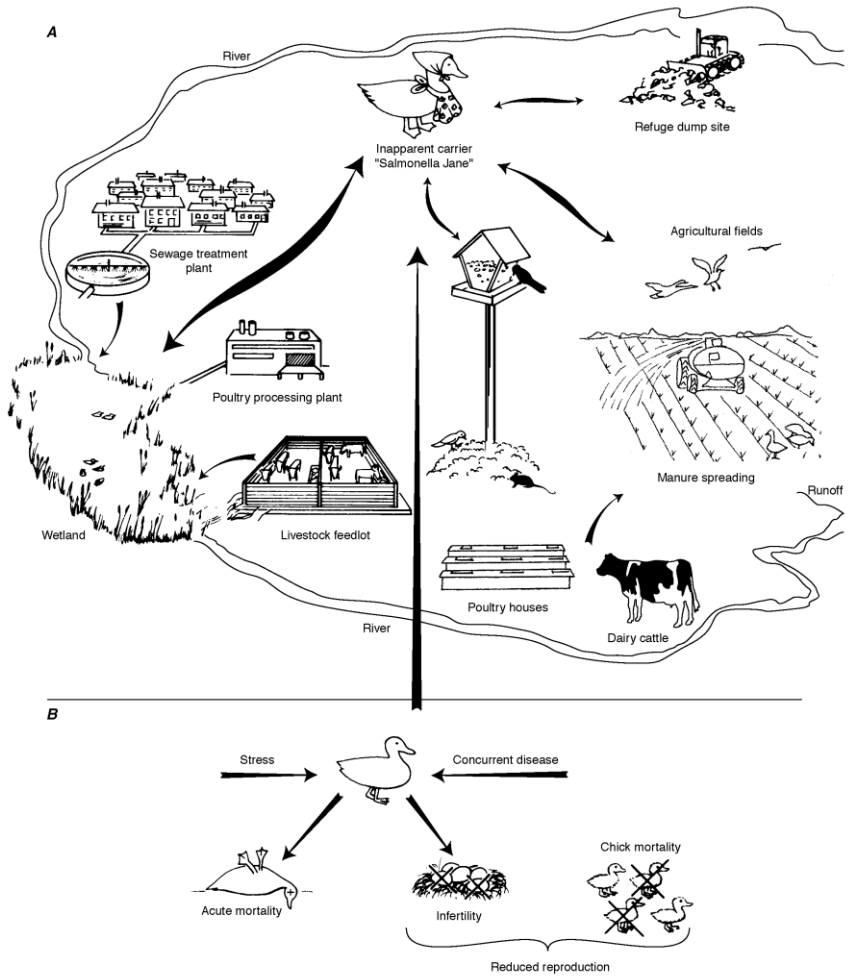

Figure 1: Sources (A) and consequences (B) of salmonellosis in wild birds. Figure from Friend, M. Field Manual of Wildlife Diseases: Birds (1999) [14]

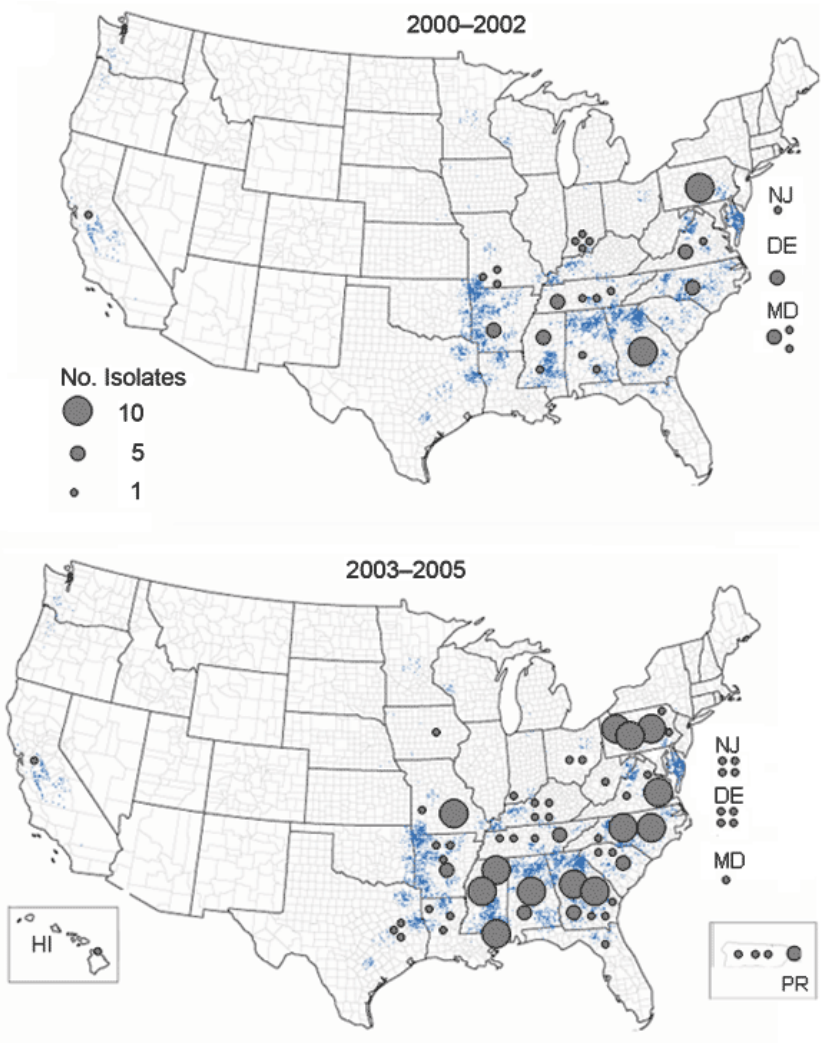

Figure 2: Geographic distribution of Salmonella Enteritidis isolates in broilers rinses in the first and second half of Altekruse et al.'s study period (2000-2002 vs. 2003-2005). Each blue dot represents 2 million broilers produced in 2002. Broiler production data: US Department of Agricultural Statistics Service. Figure from Altekruse et al. *Emerging Infectious Diseases* (2006) [17]

Table 1: Incidence (per 100,000 persons) of Human Salmonellosis in 2011 and 2012 [18, 19].

2012 Rank	State	2012 Incidence	2011 Rank	2011 Incidence
1	Arkansas	46.9	5	28.9
2	Mississippi	42.4	1	48.3
3	Florida	33.9	4	31.0
4	Louisiana	29.4	3	31.5
5	South Carolina	28.8	2	33.5
6	**Georgia**	**26.6**	**6**	**27.0**
7	Hawaii	24.6	9	24.1
8	Alabama	22.2	7	26.4
9	North Carolina	21.8	8	26.1
10	Iowa	19.8	--	14.6
	National Avg.	**16.7**		**15.7**

Table 2: Prevalence of *Salmonella* in wild caught, live passerine species for selected articles published since 1994.

Lead author	Season Captured	Location	Landscape Type	Species Collected	Samples Analyzed	Prevalence % (N)	Species Infected	Detection
Hamer[21]	Summer	Chicago, Illinois	Urban	Passerines	180	0.6 (1)	Red-winged blackbird	PCR, DNA sequencing
Gaukler[22]	Year round	Central Kansas	Livestock	European Starlings	434	0.7 (3)		Agar plate
Bradley[23]	Summer	Northern Georgia	Rural, Suburban, Urban	Passerines	247	1.2 (3)	Brown Thrasher, Carolina Wren, Northern Cardinal	Agar plate and PCR
Rogers[24]	Year round	Humboldt County, California	Livestock	Passerines	243	0	--	Agar plate
Pedersen[25]	Summer and Fall	Fort Collins, Colorado	Urban	Rock Pigeons	171	0	--	Agar plate
Pedersen[25]	Summer and Fall	Fort Collins, Colorado	Livestock	Rock Pigeons	106	8.5 (9)		Agar plate
Morishita[5]	Unknown	Ohio	Agricultural and Urban	Passerines	1709	3.9 (66)	House Sparrow, European Starling	Agar plate

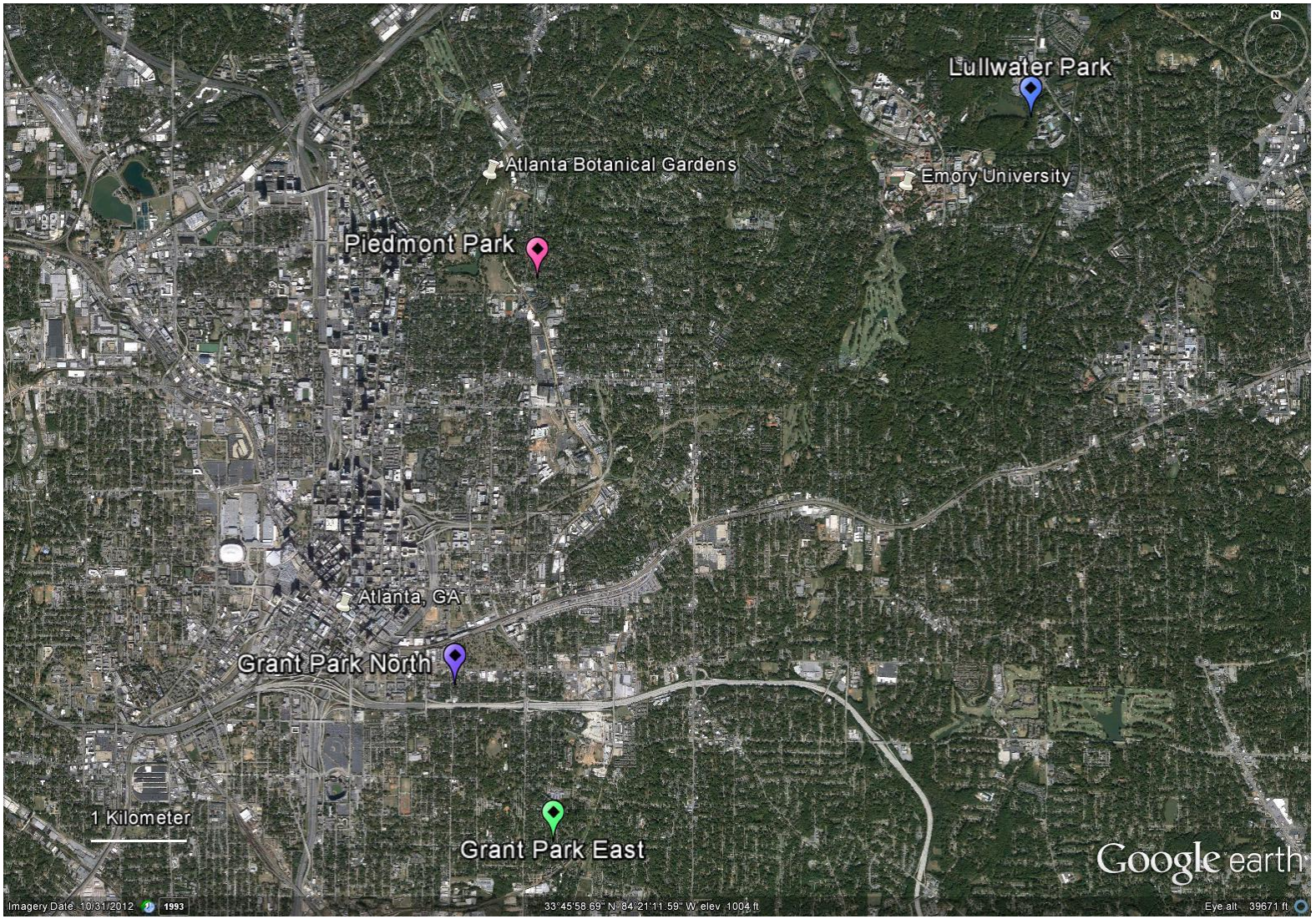

Figure 3: Location of sampling areas in Atlanta, GA, USA. Grant Park East, Grant Park North, and Piedmont areas are in Fulton County. Lullwater Preserve is in DeKalb County

Figure 4: Locations of sites within each area: (A) Piedmont Park (B) Grant Park East (C) Grant Park North (D) Lullwater Park. Cream colored points represent houses with at least 1 artificial feeding station. Images from an altitude of ~3000 feet

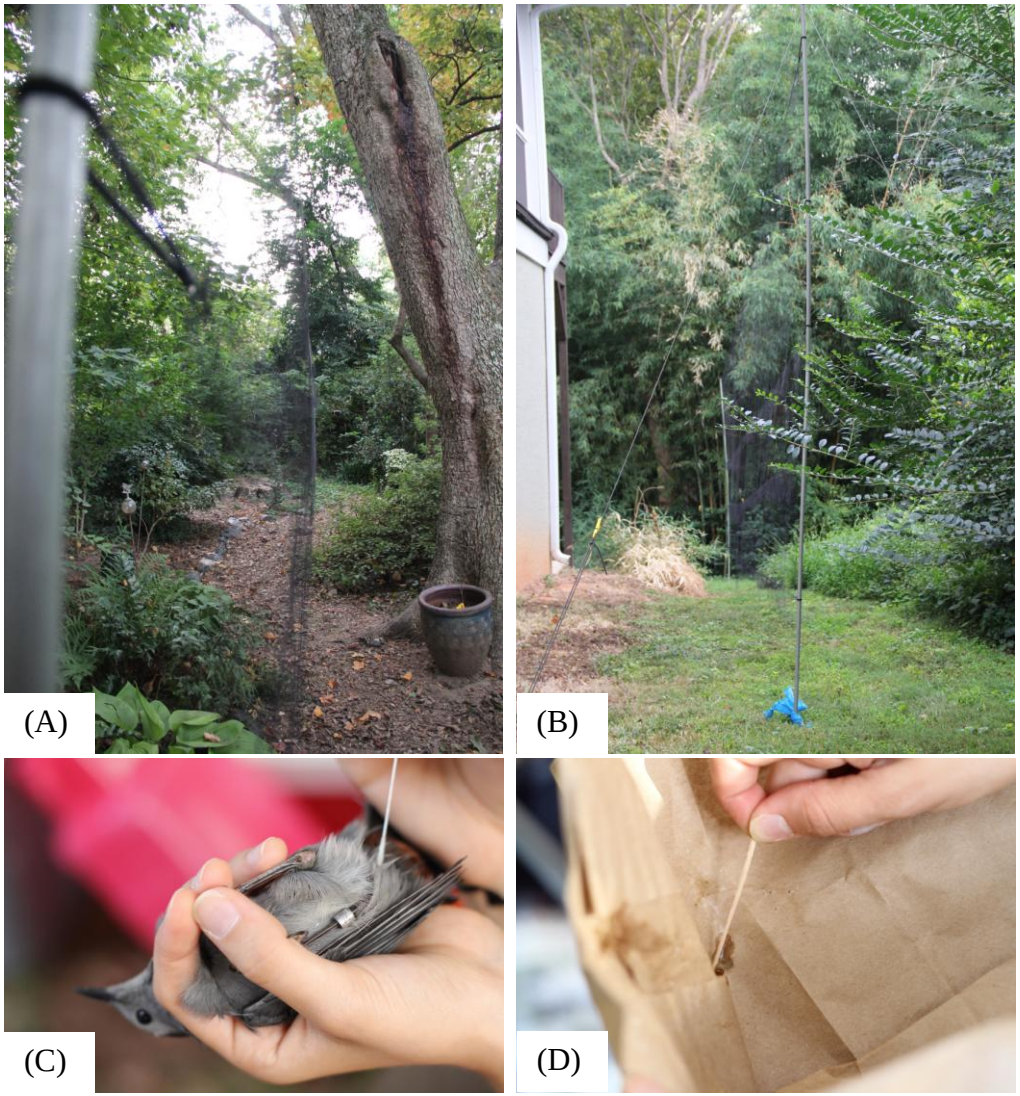

Figure 5: Photos from field collection. Mist nets set (A and B) in Piedmont area; Cloacal swab (C) and collection of feces (D). Photos by Joseph McBrayer.

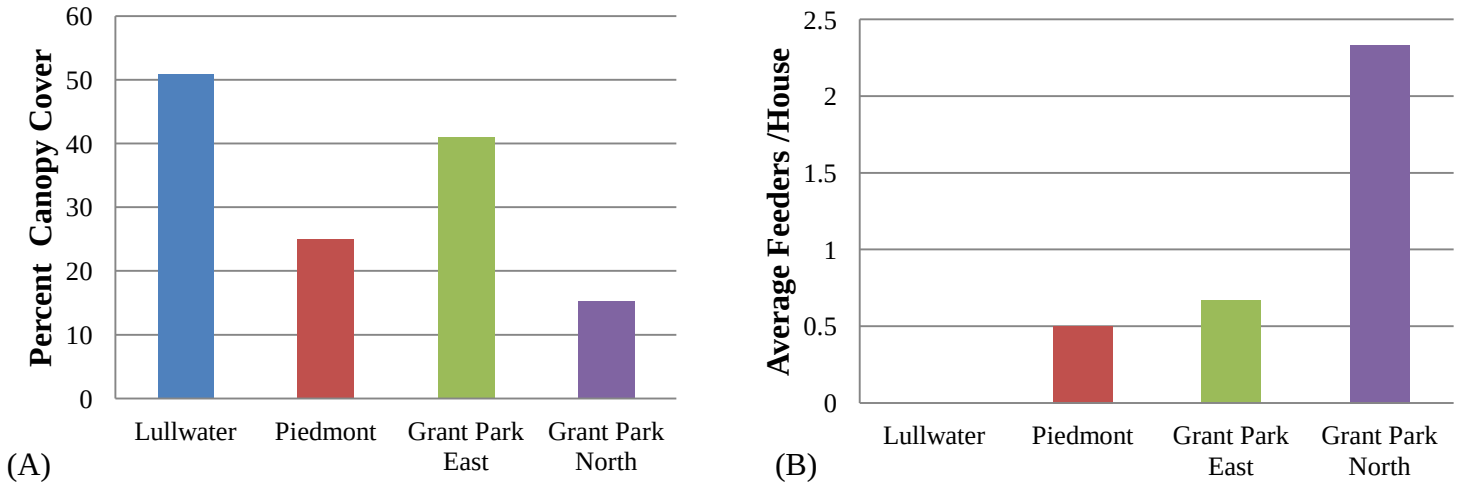

Figure 6: Percent canopy cover (A) and Average Feeders/House (B) for each area.

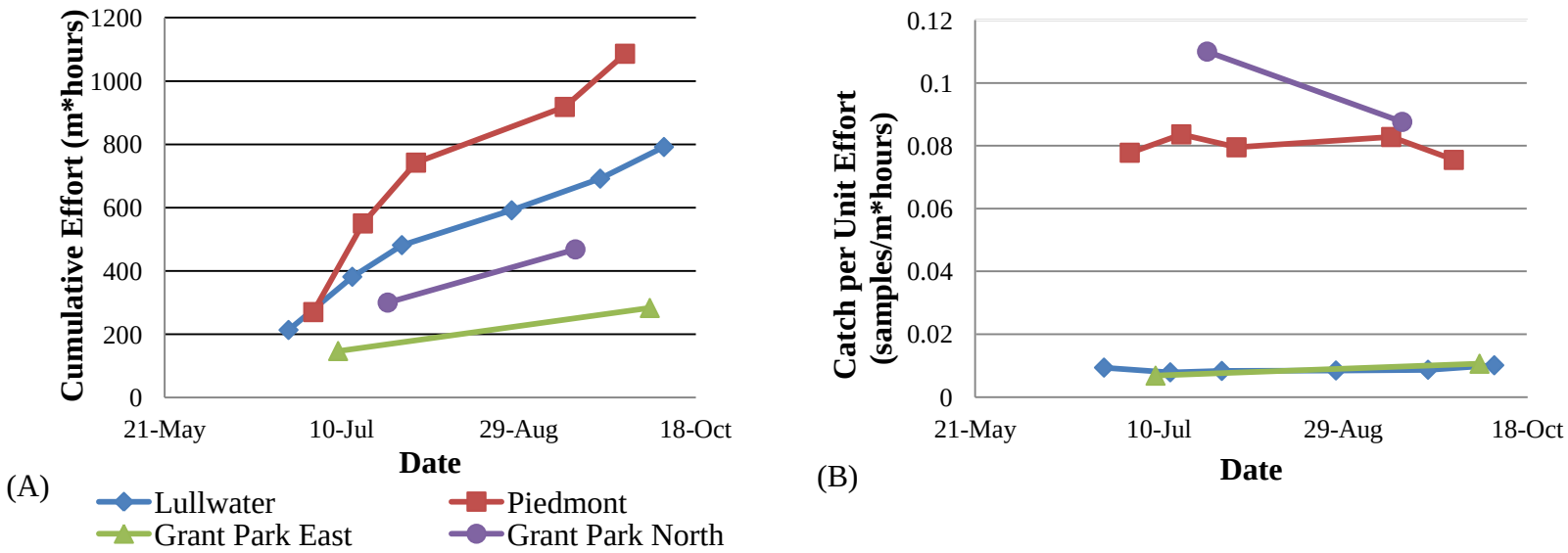

Figure 7: Cumulative Trapping Effort (A) and Catch per Unit Effort (CPUE) (B) over entire sampling period.

Table 3: Description of samples collected by site. AHY=After Hatch-Year (adult), HY=Hatch-Year (juvenile) Not reported in the table are data for the birds of unknown age and unknown sex.

Site	Bird Captures	Samples Collected N(%)	Species Richness	Age		Sex	
				AHY	HY	Female	Male
Lullwater	9	8 (5.9)	3	7	1	4	2
Piedmont	86	82 (60.7)	16	44	32	27	20
Grant Park East	4	4 (3.0)	3	4	0	1	0
Grant Park North	41	41 (30.4)	13	31	8	13	13
Total	**140**	**135 (100)**	**19**	**86**	**41**	**45**	**35**

Table 4: Species composition (N (%)) at each site.
*Represents species listed in the Top 10 Feeder Birds 2011-2012 in the State of Georgia by Project Feeder Watch [49]
Foliage=Foliage Gleaner, Ground=Ground Forager, Bark=Bark Forager, Fly=Fly Catcher

	Lullwater	Piedmont	Grant Park East	Grant Park North	Total	Feeding Behavior
American Goldfinch*	--	1 (1.2)	--	--	1 (0.7)	Foliage
American Robin	--	1 (1.2)	--	1 (2.4)	2 (1.5)	Ground
Blue Jay	--	--	--	2 (4.9)	2 (1.5)	Ground
Brown Thrasher	1 (12.5)	4 (4.9)	1 (25.0)	4 (9.8)	10 (7.4)	Ground
Brown-headed Cowbird	--	1 (1.2)	--	--	1 (0.7)	Ground
Carolina Chickadee*	--	1 (1.2)	--	1 (2.4)	2 (1.5)	Foliage
Carolina Wren*	4 (50.0)	5 (6.1)	2 (50.0)	2 (4.9)	13 (9.6)	Ground
Downy Woodpecker*	--	1 (1.2)	--	--	1 (0.7)	Bark
Eastern Towhee	--	1 (1.2)	--	6 (14.6)	7 (5.2)	Ground
Eastern Wood Pewee	--	1 (1.2)	--	--	1 (0.7)	Fly
Gray Catbird	--	6 (7.3)	--	2 (4.9)	8 (5.9)	Ground
House Finch*	--	1 (1.2)	1 (25.0)	7 (17.1)	9 (6.7)	Ground
House Sparrow	--	12 (14.6)	--	3 (7.3)	15 (11.1)	Ground
Mourning Dove*	--	--	--	1 (2.4)	1 (0.7)	Ground
Northern Cardinal*	3 (37.5)	40 (48.8)	--	8 (19.5)	51 (37.8)	Ground
Northern Mockingbird	--	1 (1.2)	--	2 (4.9)	3 (2.2)	Ground
Northern Waterthrush	--	2 (2.4)	--	--	2 (1.5)	Ground
Song Sparrow	--	4 (4.9)	--	--	4 (3.0)	Ground
Tufted Titmouse*	--	--	--	2 (4.9)	2 (1.5)	Foliage
Total	**8**	**82**	**4**	**41**	**135**	

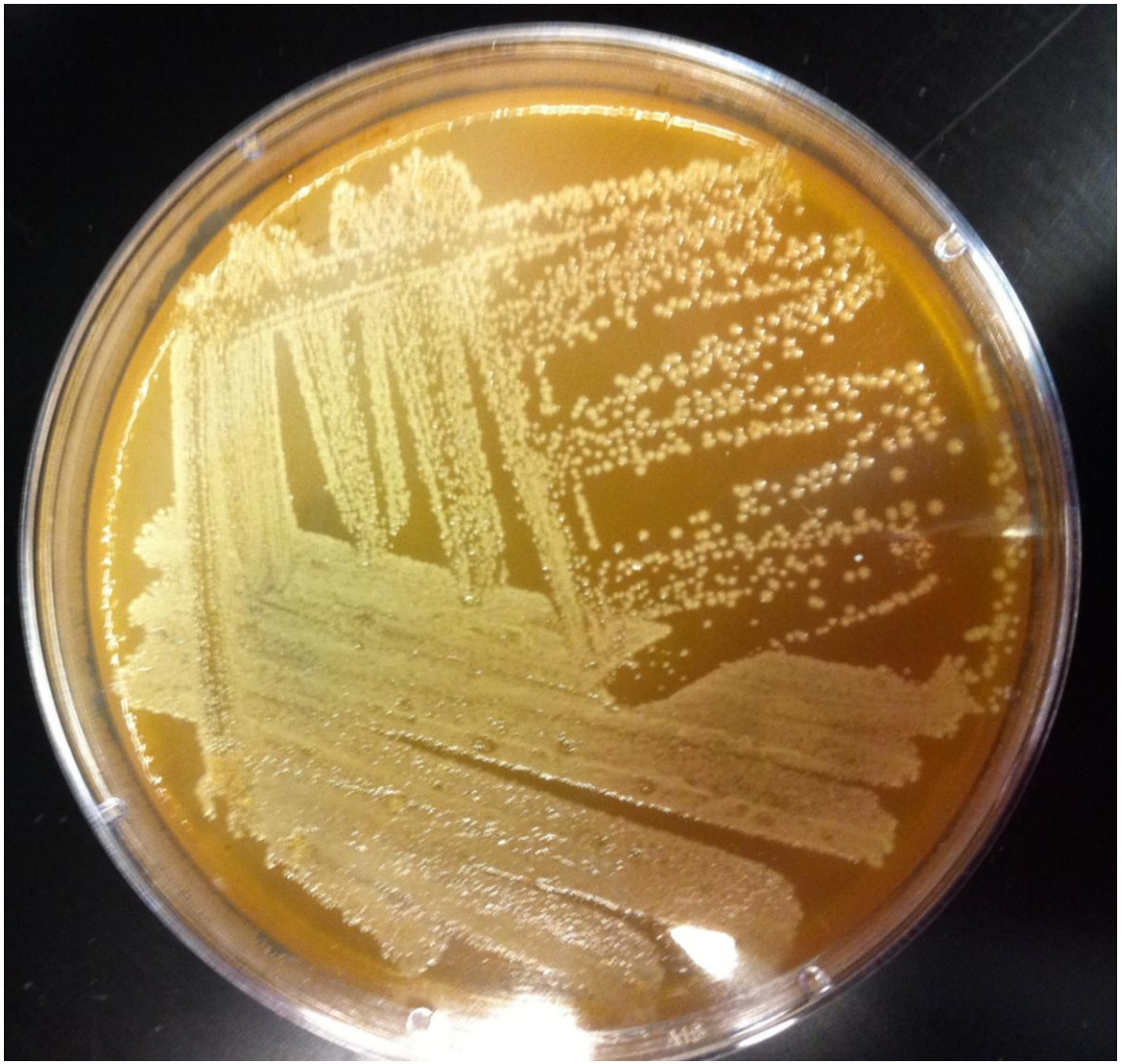

Figure 8: Gram-negative bacterial colonies growing on XLT4 agar.

Table 5: Infection Data.
*House Finches with crusty eyes suggesting *Mycoplasma* infection

Site	No. Tested	Salmonella Positive	Gram Negative Growth % (N)	No. Other Signs of Infection	No. Feeders Sampled in Yards	Feeders Salmonella Positive
Lullwater	8	0%	50.0% (4)	--	0	0%
Piedmont	82	0%	63.4% (52)	--	2	0%
Grant Park East	4	0%	50.0% (2)	--	2	0%
Grant Park North	41	0%	80.5% (33)	2*	7	0%
Total	**135**	**0%**	**67.4% (91)**	**2**	**11**	**0%**

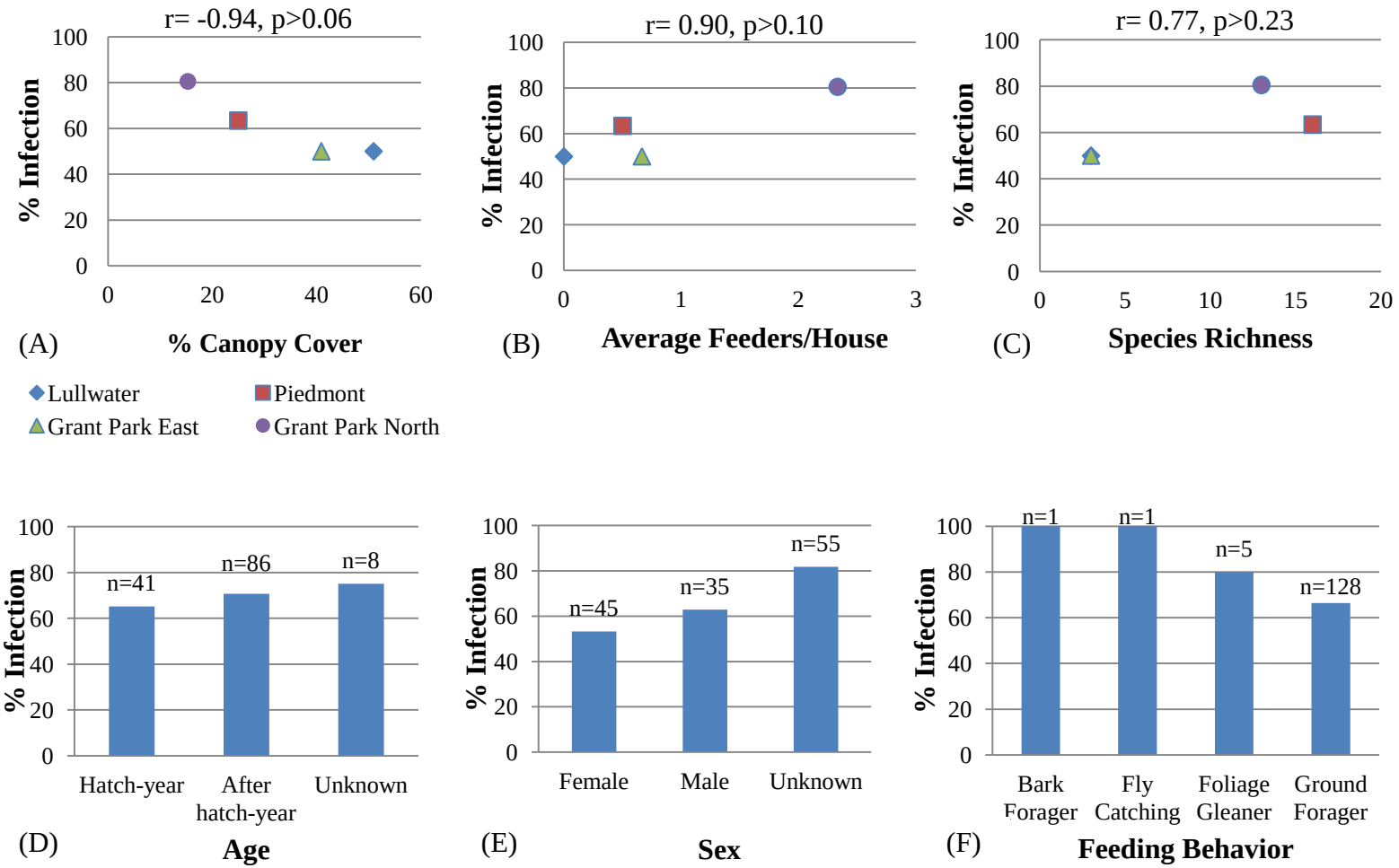

Figure 9: Gram-negative infection by landscape variables Percent Canopy Cover (A), Feeder Density (B), and Species Richness (C); Gram-negative infection by individual measures age (D), sex (E), and feeding behavior (F).

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