AVIAN INFLUENZA VIRUS AND *SALMONELLA* DYNAMICS OF NESTLING AMERICAN WHITE IBIS (*EUDOCIMUS ALBUS*)

by

KATHERINE CHRISTIE

(Under the Direction of SONIA HERNANDEZ)

ABSTRACT

Due to recent changes in breeding behavior, young American white ibis (*Eudocimus albus*) are becoming more urban-associated. We assessed the avian influenza virus (AIV) and *Salmonella* prevalence of these birds by sampling nestlings in a novel urban ibis rookery for two breeding seasons, as well as a natural rookery during one breeding season. AIV prevalence of urban juvenile ibis was evaluated, and we maintained a captive colony of nestlings to determine the characteristics of ibis *Salmonella* infection. No AIV was detected, but we found that nestlings had a high prevalence of maternal antibodies. Both free-living and captive nestlings had high prevalence of *Salmonella* with no evidence of clinical disease and reduced susceptibility to reinfection after recovery. Prevalence of *Salmonella* in urban colonies was significantly higher than natural colonies. These findings illustrate age- and habitat-specific aspects of ibis pathogen dynamics, with implications for urban wildlife ecology and public health.

INDEX WORDS: American white ibis, avian influenza virus, avian urbanization, colony-nesting birds, nestling epidemiology, *Salmonella* spp.

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KATHERINE CHRISTIE

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KATHERINE CHRISTIE

Major Professor: Sonia Hernandez
Committee: Richard Hall
Rebecca Poulson

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

The American white ibis (*Eudocimus albus*) is a nomadic wading bird common in the southeastern United States that serves as a powerful model for studying the changes wrought on a population as their natural habitat is degraded and urban zones become integrated into their ecology. By studying isolated components of this change, the epidemiology of pathogens found in breeding colonies across urban and natural populations, we can begin to piece together the many environmental and biological components impacting the health of this species. My studies build upon a history of research conducted on avian disease, urban habituation and wetland birds, and ibis-specific behavior and epidemiology as ibis begin this new chapter in their urban adaptation: the establishment of urban breeding colonies.

Urbanization and Avian Disease

Urbanization, the process of converting natural areas into developed, urban zones, is a major force impacting wildlife and ecosystems in the modern era. The changes in habitat size, quality, and composition that come with urbanization are contributing factors in massive shifts in species ranges and community structures (Jetz, Wilcove, and Johnson 2007; McKinney 2008). Within the constructed environments of urbanized zones, some species thrive while others are driven to the margins through novel and poorly understood selective processes which lead to wildlife assemblages that would not occur in natural areas (Gil and Brumm 2014; Groffman et al., 2017).

Urban wildlife populations experience a different ecological landscape than those in natural areas. Food resources may be augmented by provisioning from humans, (Galbraith et al. 2015), predator types and abundances may change (Blair 2004; Stracey 2011), and some types of environmental heterogeneity may be reduced (Luniak 2004). The presence and abundance of species is affected by habitat characteristics at the local scale and characteristics of the urban matrix itself, with greenspace areas like parks often serving as crucial hubs for urban wildlife species (Melles, Glenn, and Martin 2003). These changes all occur in different degrees depending on the level of urbanization of the area and the distribution of less-developed land that remains in the urban zone, commonly referred to as an urbanization gradient. Shifts in wildlife behavior, abundance, and other ecological traits are often measured relative to an urbanization gradient in which populations of the same or similar species are compared across habitat types defined by land cover data or other determinants of urbanization (Blair 1996). These types of studies allow researchers to detect the changes that occur in wildlife as a result of urbanization.

Avian species serve as a useful model for studying urbanization gradients. Many are highly mobile and can travel across a range of habitat types with relative ease, sometimes creating a wide array of land use combinations within a single species. Often these species frequent open developed zones such as golf courses, yards, and zoos in addition to more traditional urban spaces (Ruiz et al. 2002; Hassell et al. 2017; Kidd-Weaver et al. 2020). This creates a study system where ecological changes along an urban continuum can be detected, with minimal confounding factors related to population isolation and geography to identify the population-level effects of the urban environment on a species (Teitelbaum et al. 2020). For example, Liker (2008) indicated that morphometrics such as the tarsus length and body condition of the house sparrow (*Passer domesticus*) decreased along an increasing urbanization gradient, as measured by using building, road, and vegetation densities, in Hungary. Other studies in passerines have found that feather concentrations of contaminants such as non-essential trace elements and lead increase along urbanization gradients, studied by using populations of varying urban resource use within a single species (Roux and Marra 2007, Meillère et al. 2016).

One avian ecological process at particular risk for change in urban environments is disease ecology. Urban areas tend to be hospitable to exotic bird species (Sol et al. 2017) and so-called ‘weedy’ taxa such as ducks and pigeons known to be competent hosts for many pathogens of concern to human, wildlife, and livestock health (Krauss et al. 2004; Borges et al. 2017). This increases the opportunity for spillover transmission between species that would not typically cohabitate in undeveloped spaces. Additionally, urban wildlife populations and those that utilize provisioned food tend to gather at higher densities (Cox and Gaston 2018), and form novel interspecific interactions (Galbraith et al. 2015) than wildlife in natural areas. These population-level risk factors may be compounded by physiological stress experienced by birds in urban environments. In the rufous-collared sparrow (*Zonotrichia capensis*), the ratio of heterophils (H) to lymphocytes (L), a common indicator of chronic stress in wildlife, was higher in urban populations than rural ones, and, when rural sparrows were held in captivity, their H:L ratios resembled those of urban sparrow populations (Ruiz et al. 2002). Increased stress and changing animal behavior may cause population-level effects on disease transmission and mortality, though the presence and scale of these effects will vary between populations based on their ecological traits and level of adaptation to the chronic stress they experience (Ditchkoff, Saalfield, and Gibson 2006; Bradley and Altizer 2007). These general trends in urban community composition and density give reason to suspect that the urbanization and the environmental changes that come with it may be accompanied by major shifts in bird pathogen dynamics, which will be critical to understand in order to effectively manage wildlife in an increasingly urbanized world (Rothenburger et al. 2017).

Disease in urban avian species has the potential to affect human and livestock populations in addition to changing wildlife population dynamics and ecosystem processes. Urban areas are part of the wildlife-livestock-human interface, a term for the areas where these groups of species physically make contact and may experience conflict, behavioral changes, or disease transmission, among other things. Evidence of direct transmission of pathogens from urban birds to humans is rare, though there have been potential associations between diseases in urban avian communities and those that occur in humans during the same time period (Komar et al. 2001; Hernandez et al. 2016). Understanding the pathogen dynamics of specific wildlife communities in urban areas is critical in mitigating the risks associated with this interface, to predict or prevent future transmission events with negative economic or public health implications (Hassell et al. 2017).

The behavior and ecology of bird species affected by urbanization is changing continuously, often faster than their implications can be determined. Gaining specific understanding of these systems and their pathogens will be critical in managing the changes that are likely already occurring in the wildlife species that humans so closely associate with, especially in the ecosystems most vulnerable to degradation.

Urban Habituation of Wetland Birds

Wetlands are among the habitats most intensely impacted by human activity, including urbanization. Globally, at least 33% of wetlands have been lost in the modern age, though some estimates put this number well above 50% (Hu, et al. 2017). The economic and development trends of certain areas can lead to more concentrated loss, as seen in the state of Florida. Florida has experienced widespread wetland loss in the last century due to urban development for a rapidly expanding population and agricultural policies that lead to the dominance of sugarcane farming in regions that were once wetlands (Hefner and Brown 1984; Hollander 2009; McCauley, Jenkins, and Quintana-Ascencio 2013). Now, Florida's remaining wetlands are threatened by sea level rise (Hearn et al. 2013). These changes are of particular interest in conservation because wetlands not only provide important ecological functions such as water filtration, but also house disproportionate amounts of biodiversity within their land area (Gopal and Junk 2000). Wetlands are often drained and filled to create land area suitable for agriculture or urban development, displacing the many specialized species that reside there.

Though most species are likely to experience population declines as a result of habitat loss, wetland bird species may be particularly sensitive. One study in Shanghai, China showed that wetland birds experienced the largest losses after a habitat disruption event impacting birds of many land use types (Xu et al. 2018). The mechanism for this trend is unknown and thus cannot be generalized to all wetland birds at this time, but factors that may increase wetland bird vulnerability include the large body size of wading birds, specialized food sources, and complex nesting requirements. Wetland bird population counts correspond closely to the amount of wetlands remaining at a landscape scale, indicating these populations require large amounts of minimally fragmented wetland area (Quesnelle, Fahrig, and Lindsay 2013). Additionally, some wading bird species rely on specific cycles of hydrologic inundation in order to find food, and these cycles can be detrimentally altered by land development and sea level rise (Murray et al. 2018; Yilmaz et al. 2020). Colony nesting is common among wetland bird species and is an ecologically sensitive process, with changes in water flows and habitat quality associated with huge variations in colonial nesting occurrence and success in Florida and elsewhere (Frederick and Collopy 1989; Kelly et al. 2008; Brandis et al. 2018). Thus, the loss or alteration of wetland habitats may have extreme effects on avian species, expediting their incursion into urban areas and the accompanying changes to their ecology.

Studies of urban-habituated wetland birds have identified behaviors and health characteristics associated with urban populations. Few, however, have directly compared these to natural populations to determine whether urban resource use causes population-level changes in the birds. Comparisons of populations of species such as the wood stork (*Mycteria americana*) and cattle egret (*Bubulcus ibis*) along urbanization gradients have revealed some urbanization-based trends in wetland birds, such as toxin load differences and reproductive rate variation (Yasmeen et al. 2019; Evans and Gawlik 2020). Some work has also compared urban and natural populations of Australian white ibis (*Threskiornis molluca*) populations. In Australian white ibis, these gradient-based studies have been made possible by the recency and speed of their urbanization, with the ibis reaching pest status in urban zones over the last three decades as their natural wetland habitats and populations have dwindled (Kingsford and Thomas 1995; Smith 2009).

A similar series of events is occurring in the American white ibis. The American white ibis (hereafter referred to as "ibis") is a nomadic wading bird common across the Southeastern United States, where it is closely associated with wetlands and other aquatic environments. In Florida, where development and climate change threaten the already-reduced wetlands, ibis have become a common sight in urban parks and backyards in addition to their natural habitats (Frederick et al. 1996; Kushlan 2011; Hernandez et al. 2016). As with other wading birds, changes to wetlands have the potential to drastically affect this species. Ibises are tactile foragers that feed on a variety of aquatic invertebrates and small fish. The depth at which they forage varies based on the type of prey, but crayfish, their most important food source, seem to be found most efficiently at about 29 cm of water depth, and sizeable increases in depth have caused ibises to abandon some inland marshes (Kushlan 1979; Binkley, Dorn, and Cook 2019). Alterations of the hydrologic cycles of inundation in wetlands caused by upstream development or sea level rise will likely affect ibis and other wading birds in Florida, reducing the suitability of the wetlands that remain to them (Murray et al. 2018; Yilmaz et al. 2020). Already, these changes have massively reduced Florida's populations of wetland wading birds and crippled the ecosystem's resilience to threats such as invasive species and drought (Walker and Solecki 2004). Ibis productivity has been reduced in other American wetlands (Trexler et al. 2009; Dorn et al. 2011) and they have been driven to utilize urban areas where food can be reliably found by both foraging and human provisioning (Murray et al. 2018 and. 2020). The dual processes of habitat loss and urbanization have altered the species' movement patterns and behavior (Welch 2016; Kidd-Weaver et al. 2020), with ecological implications including changes in pathogen prevalence and transmission (Hernandez et al. 2016).

Florida's ibis populations provide a rare opportunity to study a species currently going through the process of urban adaptation, with novel behaviors and ecological patterns still emerging. The ongoing study of American white ibis has revealed urbanization-associated changes in movement, diet, and health in adult ibis. In one study using GPS backpacks to track ibis movement and behavior, it was found that those that utilize more urban resources tend to have shorter, earlier breeding seasons and ibis with intermediate or high urban association were more likely to skip the dispersal and breeding seasons altogether (Kidd-Weaver et al. 2020). Other studies have indicated that the urban ibis diet has shifted, with accompanying health tradeoffs. Those that utilize more urban areas and assimilate more provisioned food tend to have lower body condition scores and decreased gut microbiome community diversity but also lower ectoparasite loads (Murray et al. 2018; Murray et al. 2020), and these urban populations have also been associated with increased *Salmonella* prevalence and shedding rates (Hernandez et al. 2016; Murray et al. 2020). Recent changes in nesting sites indicate that their reproductive habits in relationship to urban areas are currently developing, making them an ideal model for detecting changes along that gradient (Hernandez pers. comm). Young ibis age classes are relatively understudied, partially due to the challenges in accessing the large wetland colonies where ibis tend to breed. The novel occurrence of breeding colonies within urban zones opens a new route by which urbanization could impact ibis populations.

Urban Avian Breeding Colonies

During the breeding season, when the need for consistent freshwater prey availability is greatest, ibis historically return to large, natural wetland breeding colonies with as many as 60,000-100,000 individuals (Kushlan 1977). Continued wetland degradation could limit the ability of natural areas to support this part of the ibis life cycle in addition to their already-altered foraging behavior (Frederick et al. 2009). The resultant large-scale increase in the utilization of urban zones for reproduction could change characteristics of the ibis breeding season, with the capacity to shift pathogen dynamics amongst immunologically-naïve nestling populations which previously had not been part of the urban ibis community. As of now, a small number of active urban rookeries have been identified in Florida (Welch 2016; Hernandez pers. comm.). The development of these rookeries heralds a major change in the environment that some ibis nestlings and young juvenile ibis are exposed to and raises questions about how the shifts in health and pathogen prevalence that accompany adult urbanization will translate into the younger parts of the population.

Some of these unknowns have been studied in other urbanizing bird species that historically bred in colonies in undeveloped landscapes (Tella, Hirlado, and Donazar-Sancho 1996; Pierotti and Annett 2001; Smith 2009; Kamp et al. 2015). Changes in breeding success are not unidirectional, however, as metrics influencing nestling survival such as food availability, nest predation, and hatchling health vary depending on the needs of the species and the urban environment that they have entered. For example, the lesser kestrel (*Falco naumanni*) in Spain had decreased breeding success in urban colonies due to high levels of nestling starvation (Tella, Hirlado, and Donazar-Sancho

1996), whereas the Northern Lapwing (*Vanellus vanellus*) in Germany had increased nestling survival in urban breeding colonies, attributed to lower predation (Kamp et al. 2015). The Australian white ibis (*Threskiornus molucca*) experienced increased fledging success per clutch in urban rookeries compared to those in natural areas (Smith 2009), though there was high variability between breeding sites and the causes of this change in fledging success are poorly understood.

In all of these cases, nestlings are exposed to the landscape outside of the rookery through their parents. The effects of the urban South Florida landscape on the health of American white ibis nestlings are unknown, and the shift from natural to urban breeding changes characteristics of the ibis rookery beyond just the direct impacts of the landscape such as parental diet and environmental contamination. Colony size is one such characteristic, as urban colonies tend to be smaller than their natural wetland counterparts. One study found that colony size impacted the immune response of common tern (*Sterna hirundo*) nestlings, with immune response decreasing and physiological stress, measured using H:L ratios, increasing with colony size (Minias et al. 2019). This illustrates that, in colony-nesting birds, nestling stress may be lower in smaller colonies. Urban rookeries, however, experience additional changes that add complexity to this trend. The increased noise levels in urban zones have been found to increase oxidative stress levels in the tree swallow (*Tachycineta bicolor*) (Injaian, Taff, and Patricelli, 2018) and increase acute corticosterone response in the western bluebird (*Sialia mexicana)* (Kleist et al. 2018), making stress levels in urban ibis breeding colonies difficult to predict.

Urban ibis nestling health metrics and disease may be impacted by altered pathogen prevalence in addition to changes brought on by their changing stress levels, diet, and contaminant exposure in the rookeries. These interacting urban health and environmental characteristics must be evaluated in specific pathogens in young ibis to better gauge the resultant changes in disease ecology as the species' reproductive processes move into urban areas. The survival pressures on this vulnerable age class are poorly understood, and the potential for urbanization-induced pathogen prevalence changes brings added urgency to filling in these gaps in life history and ecological knowledge that may be indicative of larger trends in wetland bird populations.

Salmonella in Ibis

One pathogen of interest within the urban ibis breeding colony is *Salmonella* sp. *Salmonella* is a genus of Gram-negative enterobacteria that is divided into two species, multiple subspecies, and over 2500 serotypes. In wild birds, *Salmonella* infection is relatively common and is typically spread through exposure to contaminated surfaces or water or through the consumption of contaminated prey (Tizard 2004). It is considered an emerging disease in songbirds, causing large outbreaks and major mortality events often associated with congregations at bird feeders (Plant 1978; Tizard 2004). In many other species, however, the pathogen does not typically cause clinical disease or mortality in healthy adults, though the young, old, or immunologically compromised may be more affected (Hilbert et al. 2012; Friend 1999; Tizard 2004). Wild adult ibis have been found to be competent hosts for a high diversity of *Salmonella* serotypes and genotypes and evidence indicates that birds are frequently infected, recover, and then become reinfected with different *Salmonella* serotypes (Hernandez et al., 2016). Though it is not usually considered a population-level threat to wading birds such as the ibis, studying *Salmonella* epidemiology in these birds sheds light on how this pathogen is maintained in the population, the sublethal ways that it may affect the ibis, potential impacts on the surrounding wildlife community and public health, and the changes to pathogen dynamics that occur alongside the urban adaptation of these birds.

Ibis that forage in urban areas and assimilate anthropogenic food tend to have lower gut microbiome diversity than ibis in natural areas (Murray et al. 2020). This lower microbiome diversity is associated with increases in *Salmonella* shedding amongst ibis, indicating the urban ibis diet could potentially facilitate increased *Salmonella* transmission (Murray et al. 2020). Overall *Salmonella* prevalence was also higher among adult ibis in urban areas versus natural ones, as measured by the positive correlation of *Salmonella* prevalence and open-developed landcover common in parks and golf courses (Hernandez et al. 2016). This indicates that urban landscapes support a higher prevalence of *Salmonella* than the ibis' natural habitat, likely due to a combination of factors including the characteristics of the ibis' urban foraging sites.

Urban ibis typically forage in parks and yards where they are sometimes provisioned with bread and other processed foods, causing them to gather in large flocks for extended periods of time. They often share these sites with known *Salmonella* reservoirs such as ducks and may also be exposed to contaminated human sewage and refuse. In this already risk-filled environment, these dense ibis groups can further contaminate the site with feces on the surfaces and in the water. The use of these sites can lead to increased transmission of *Salmonella* compared to natural foraging behaviors, especially when compounded by dietary changes that increase shedding rates. A similar effect of foraging site on *Salmonella* prevalence in an urban population has been identified in herring gulls (*Larus argentatus*), another aquatic bird species that serves as a *Salmonella* reservoir (Monaghan et al. 1985; Palmgren et al. 2006). *Salmonella* prevalence of herring gulls within an urban area was associated with their foraging site type, higher in populations that foraged near sewage outputs versus landfills. These studies on ibis and other aquatic birds demonstrate the decisive impact that changing wildlife behaviors within an urban landscape can have on pathogen prevalence. In adult ibis, the urban transition has altered the prevalence of *Salmonella* in the populations, though the implications of this are not yet fully understood. These birds do not commonly develop clinical disease, but sublethal effects or complications from other components of the urban environment are possible, in addition to potential connection to human *Salmonella* cases (Hernandez et al. 2016).

The effect of *Salmonella* on nestling ibis, especially those in urban zones, is also only beginning to be explored. The same study that found ibis' urban land use association with *Salmonella* also found that, by site, the *Salmonella* prevalence of nestling ibis is higher than that of adults (nestlings 35%, N= 72, adults 13%, N= 261), though this study only included nestlings from breeding colonies in natural areas (Hernandez et al. 2016). This is not surprising, as nestlings are immunologically naïve, but it raises questions about how this effect will be exacerbated in the urban rookery, with the already-elevated pathogen prevalence of urban adult ibis. Urban-bred nestlings are provided their diet by parents that are likely foraging in areas such as landfills, parks, and golf courses, leading to potential poor nutrition and exposure to infected adults. Additionally, these nestlings reside in dense, mixed-species breeding colonies and experience the previously described potential changes in colony dynamics caused by the urban matrix. These combined effects could lead to both elevated pathogen prevalence in nestlings as well as increased risk of disease during infection. The environmental conditions of the rookery as well as nestling exposure to potentially *Salmonella*-positive urban adult ibis and other bird species could cause a higher prevalence of *Salmonella* in the nestlings, while their likelihood of expressing clinical disease may be increased by urbanization-induced changes in diet and stress. Further study on the characteristics of nestling ibis *Salmonella* infections as well as pathogen prevalence in ibis rookeries in both urban and natural settings is needed to better understand these potential epidemiological changes in urban breeding colonies, the newest frontier in the study of ibis, their pathogens, and their environment.

Avian Influenza Virus (AIV) in Ibis

The other pathogen of interest in this study of urban ibis breeding colonies is avian influenza virus (AIV). AIV are negative-sense single-stranded RNA viruses of the family Orthomyxoviridae. This subset of Type A influenza viruses is adapted to and maintained in birds, though some can infect mammals, including humans. Within an influenza type, viral subtypes are identified by their combination of hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. The reassortment of these proteins creates variable subtypes which are maintained in some avian populations, with subtypes HA1 through HA16 and NA1 through NA9 of type A influenza virus having been detected in birds. Avian species likely serve as the ultimate genetic reservoir from which influenza in other taxa arises (Webster et al., 1992). These reassortment events which create immunity-avoiding new influenza strains occur by chance, and thus are more likely to occur when influenza prevalence is high and a high diversity of hosts and viral subtypes are present.

AIV is often the target of wildlife disease surveillance efforts due to the rapid evolution of the pathogen and its zoonotic potential. Waterfowl and shorebirds are the most common species of interest in understanding AIV in natural populations, though more hosts are continually being identified (Webster et al. 1992, Stallknecht and Brown 2017). Outbreaks of AIV are often associated with large congregations of birds, such as the wintering grounds of migratory waterfowl. These outbreaks can cause lethal and non-lethal cases and sometimes spillover to poultry operations, with significant economic effects. The shedding rates, seasonal prevalence patterns, and transmission likelihoods vary widely among natural hosts, however, leaving many gaps in our understanding in AIV maintenance in wild birds (Stallknecht and Brown, 2017; Tyson-Pello and Olsen 2020). A full understanding of AIV epidemiology in wild birds thus requires study of the unique components of the host-pathogen relationship in many host species.

Compared to the knowledge of the ibis-*Salmonella* system, understanding of AIV in ibis is less complete. Ibis are susceptible to experimental infection with AIV and are infected in natural populations, as evidenced by the detected seroprevalence of AIV antibodies of over 70% (Bahnson et al., 2020). Ibis have many ecological characteristics that indicate they could be part of the natural AIV cycle, such as their association with aquatic environments, colonial breeding, and, critically, their nomadic nature and co-occurrence with known AIV reservoirs such as ducks and gulls. The timing of their involvement in this cycle is unknown, however, because although many ibis with AIV antibodies have been identified, ibis with active AIV infections have not. This temporal information has proven difficult to attain in ibis, partially due to the short shedding period of AIV detected during experimental infection trials (5 days for H11N9, 6 days for H6N1), which is a relatively common barrier in AIV research (Webster et al. 1992; Bahnson et al. 2020). As ibis spend large portions of their lives near important maintenance hosts and have been known to travel to critical avian gathering areas, such as regions on the Mississippi flyway (Kidd-Weaver et al. 2018), how frequently and for what duration they are infected with and shed AIV is a crucial detail in determining their role in the natural AIV cycle.

AIV epidemiology in ibis is also particularly relevant because of their growing association with urban environments. Changes in pathogen prevalence associated with this urban adaptation have already been found with *Salmonella* (Hernandez et al. 2016) and whether this pattern applies to AIV is an open question. Many of the characteristics of ibis that make them a potential player in the larger AIV cycle are made more evident in urban zones, where they cohabitate with waterfowl and shorebirds in higher density foraging and breeding sites and have biological and behavioral shifts that may change their health (Bahnson et al. 2020; Murray et al. 2020). In developed areas such as cities, AIV dynamics are changing (Vandegrift et al. 2010; Wu et al. 2017), with altered avian behaviors and resource availability paired with novel and potentially harmful community assemblages, making understanding the timing of AIV infection in a prolific urbanizing bird such as the ibis an imperative.

The occurrence of urban breeding colonies thus adds a new layer to this concern. Many of the health-related risk factors discussed in relation to *Salmonella* in urban breeding colonies, such as diet changes, high densities, and stress, apply here as well, with the added knowledge that young birds play a critical role in AIV maintenance in some important host populations, such as juvenile mallards (*Anas platyrhynchos*) (Stallknecht and Brown 2017). In nestlings, additional factors such as the presence of maternal antibodies to AIV, which have been found to be closely related to adult AIV antibody seroprevalence in other colony-nesting aquatic birds (Pearce-Duvet et al. 2009; Hammouda et al. 2011; van Dijk, Mateman, & Klaassen 2014), complicate the backdrop on which these urban changes may occur. Understanding the general relationship of nestling and juvenile ibis to AIV and the specific patterns emerging as their urbanization continues fills in crucial gaps in the existing knowledge of this host-pathogen system.

Purpose and Significance

The objective of this work is to expand the epidemiological information on white ibis by studying specific pathogen dynamics in young ibis in different environments, particularly focused around novel urban breeding colonies. Adults of the species have been extensively studied, providing a rich contextual background for the analysis of nestling and juvenile epidemiology. By focusing on these age classes in detail, these chapters contribute to an already exceptionally thorough, full life cycle understanding of host-pathogen relationships in this avian species. This holds particular significance due to their status as an urbanizing aquatic bird, allowing us to track how environmental and anthropogenic changes affect different components of their disease ecology.

In Chapter 2, I analyze *Salmonella* prevalence and diversity in nestling ibis in multiple environmental contexts, allowing us to better understand the progression and characteristics of *Salmonella* infection in nestling and juvenile hosts and how this may affect ibis populations as their breeding location shifts. This was done by maintaining a captive colony of nestling ibis and tracking their *Salmonella* status under captive conditions, including experimental infection after maturation to the juvenile stage. We also sampled free-living ibis nestlings in both urban and natural rookeries repeatedly over multiple weeks to determine patterns in prevalence in wild nestlings in different habitats. I hypothesized that *Salmonella* prevalence would be relatively high in all sets of birds due to the high density of both the captive conditions and breeding colonies and the high *Salmonella* prevalence found in adults. I also hypothesized that prevalence would decrease with age, due to the immune naivety of nestlings, and that early infections may lead to some resistance to infection in older nestlings or young juveniles. I considered it likely that there may be occurrences of clinical disease and death in infected young birds. Additionally, I predicted that the wild nestlings in urban breeding colonies would have higher *Salmonella* prevalence than the natural colonies and have *Salmonella* serotypes associated with urban zones, due to this pattern being found in adults in previous studies. This age-specific data adds new layers to our understanding of the ibis-*Salmonella* system, making it one of the most detailed bodies of work on *Salmonella* in a wild bird species.

In Chapter 3, I analyze the prevalence of AIV in nestling and juvenile ibis in urban areas, repeatedly sampling nestlings in urban and natural colonies during their first three weeks of life and opportunistically sampling juveniles in urban parks during the winter influenza season. Previous studies of adult white ibis have indicated that birds are commonly infected in the wild and are competent hosts for multiple AIV strains, but the timing and frequency of these infections is unknown. Influenza viruses are difficult to detect due to their short shedding window, and thus necessitate the investigation of specific hypotheses. For ibis, these hypotheses are as follows: 1) ibis are frequently infected with and recover from AIV throughout their lives, leading to the high natural seroprevalence of AIV antibodies found in natural populations, or 2) ibis are commonly infected with AIV during a specific life stage, after which they recover and seroconvert, leading to persistent antibodies which account for the high adult seroprevalence. For the latter hypothesis, both nestling and juvenile ibis have been considered viable candidates for the age group in which infection commonly occurs, nestlings due to their immune naivety and the dense conditions of breeding colonies and juveniles due to their continued relative immune naivety and novel independent environmental exposures. The concentrated study of these age groups could determine whether this age-group-dependent timeline theory is the answer to questions surrounding ibis influenza epidemiology and potential risks. Additionally, if young ibis are commonly infected with AIV, then this shift in breeding behavior that leads to more ibis nestlings and young juveniles being exposed to the urban landscape has the potential to change the epidemiology of AIV in ibis.

We completed longitudinal sampling of nestlings, testing for influenza infection as well as maternal antibodies. Fecal samples from nearby urban juveniles up to 3 years old were tested for active infection. I hypothesized that nestlings may be infected with AIV due to their cohabitation with known AIV reservoir species in breeding colonies, with higher prevalence in the urban colonies. Alternatively, I hypothesized that if nestlings had high levels of maternal antibody protection then virus prevalence could be low, with juveniles instead being the most vulnerable, as these protections dwindled. This study investigated these proposed timelines of infection related to young ibis, helping fill in gaps in understanding around how and when adult ibis are infected with and shed AIV, a pathogen of ecological, economic, and public health concern in urban areas.

By completing these monitoring and experimental procedures accounting for many components of the host-pathogen relationship, this study establishes benchmark values for pathogen prevalence and dynamics within understudied ibis age groups and sheds light on the differences between urban and natural populations of a species whose ecology makes it particularly vulnerable to anthropogenic changes and disease.

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CHAPTER 2

SALMONELLA DYNAMICS IN AMERICAN WHITE IBIS (*EUDOCIMUS ALBUS*) NESTLINGS

Introduction

Salmonella sp.

Salmonella is a genus of Gram-negative enterobacteria that has a wide host range, but in humans causes gastrointestinal symptoms. The genus is divided into two species. One of these, *S. enterica*, is further divided into subspecies and over 2500 serotypes, differentiated by characteristics of their surface structures such as fimbriae, flagella, and polysaccharides. The Centers for Disease Control and Prevention (CDC) currently estimates that 1.35 million people in the United States are infected with *Salmonella* annually (CDC 2021), and *Salmonella* infections contribute to the approximately $90 billion spent on foodborne illnesses in the United States each year (Scharff 2018; Scharff 2020). The detection and identification of *Salmonella* has well-established methods. There are over 340,000 published *Salmonella* genomes, most of which were added in the last 10 years, which can be used to track the source of *Salmonella* outbreaks (Brown et al. 2021). *Salmonella* in humans is often attributed to contaminated meat, particularly chicken, and the poultry industry invests additional billions of dollars in the prevention, treatment, and management of *Salmonella* outbreaks (Crim et al. 2015; Atterbury et al. 2020), though other transmission routes involving wild animals, including birds, have been identified (CDC 2007; Jain et al. 2019).

Some serotypes, such as *S.* Enteritis and *S.* Typhimurium can infect a broad range of hosts, while other subspecies are limited to a single host species. The variety in pathogenicity amongst serotypes comes from how the surface structures and genetics of the bacteria interact with the within-host environmental conditions, such as the pH, the community of commensal bacteria such as *Escherichia coli*, and immune response (Foley et al. 2001). As summarized by Foley et al., ingested salmonellae must survive the low-pH environment of the host stomach and then pass into the lumen of the gastrointestinal (GI) organs, where they compete with other gut bacteria to adhere to epithelial cells (Foley et al. 2013). Once adhered, many *Salmonella* sp. rely on type III secretion systems (T3SS), to invade cells. A T3SS, a surface protein structure encoded by *Salmonella* pathogenicity island 1 (SPI-1) facilitates the injection of bacterial effector proteins into the host cell by rearranging the host cytoskeletal system. This leads to the expansion of the host cell membrane and the engulfment of the *Salmonella* cell, packaged in a *Salmonella* containing vacuole (SCV), in a process called membrane ruffling (Lostroh and Lee 2001; Andrews-Polymenis et al. 2010; Foley et al. 2013). Another T3SS, encoded by SPI-2, injects further effector proteins into the host cytoplasm which lead to the formation of *Salmonella* induced filaments (SIFs) which protrude from the SCV into the space of the host cell and contribute to bacterial pathogenicity and possibly to *Salmonella* replication.

The progression to a severe case of salmonellosis in the host relies on the successful invasion of macrophages, macrophage-like cells, or dendritic cells, which allows the infection to spread (Foley et al. 2013). The method of invasion, however, varies between and within serotypes, with between-strain distinctions in genetic makeup indicating many strains within a serotype are adapted to specific hosts (Hughes et al. 2008; Foley et al. 2013). Infected hosts often shed *Salmonella* intermittently in their feces, and *Salmonella* can pass through the GI tract and into feces even if the host intestine is not colonized by the bacteria (Andrews-Polymenis et al. 2010; Foley et al. 2013). In birds, a diagnosis of lethal salmonellosis can be differentiated from incidental *Salmonella* infection during necropsy, using histopathological analysis or tissue culture, often focusing on the crop, liver, and spleen, where *Salmonella* tends to multiply in severe infections, causing enlargement, discoloration, lesions, and other signs (Alley et al. 2002; Pennycott, Park, and Mather 2006; Hughes et al. 2008).

The Significance of Salmonella in Birds

Infection with *Salmonella* sp. is common in many wild birds, either through consumption of infected prey or exposure to contaminated surfaces or water (Tizard 2004). Disease and mortality associated with *Salmonella* sp. infection have the potential to cause population level effects in some hosts and are a major motivation for studying this pathogen in wild birds. Passerine species across the world have experienced major mortality events as a result of *Salmonella* outbreaks, often attributed to congregation at bird feeders (Kirkwood and Macgregor 1998; Hall and Saito 2008; Lawson et al. 2018). *Salmonella* outbreaks in passerines have been on the rise in recent decades and can be considered an emerging disease associated with anthropogenic activity (Plant 1978; Tizard 2004).

In one study of bird mortalities reported by an animal hospital in Scotland between 1995 and 2003, carcasses representing 32 passerine and non-passerine bird species were analyzed to determine whether *Salmonella*, specifically *S*. Typhimurium, was a significant cause of death (Pennycott, Park, and Mather 2006). They found salmonellosis as a cause of death in individuals from 15 of these species, and it was considered a significant cause of death in 3, all of which were passerine species. It caused 72% of mortalities (N= 190, 35 sites) in the greenfinch (*Carduelis chloris*), 66% (N=50, 13 sites) in the house finch (*Passer domesticus*), and 28% (N=53, 9 sites) in the chaffinch (*Fringilla coelebs*). In another study, 21.5% of passerine mortalities recorded in the US Geological Survey (USGS) National Wildlife Health Center database from 1985 to 2004 were attributed fully or partially to salmonellosis (Hall and Saito 2008). This demonstrates the potential population level impacts of *Salmonella* in birds, particularly passerines.

In other avian groups, mortality is far less common but has been found in the young, old, or immunologically compromised in some species, and many potential sublethal effects remain uninvestigated (Friend 1999; Clavijo, Robinson, and Lopez 2001; Tizard 2004; Une et al. 2008; Hilbert et al. 2012). These impacts have the capacity to significantly affect populations in situations where transmission risk is high, such as in breeding colonies. There have been instances of high-mortality *Salmonella* outbreaks in non-passerine avian breeding colonies, usually when individuals are co-infected with another pathogen such as Newcastle Disease Virus (NDV) (Clavijo, Robinson, and Lopez 2001; Tizard 2004; Hall and Saito 2008).

Due to the significance of *Salmonella* to public health and the poultry industry, its potential transmission to and from wild birds is another motivation for the study of this pathogen. Using the comparison of genetic relatedness with published *Salmonella* genomes, and other epidemiological methods to locate the sources of outbreaks, studies have revealed that some *Salmonella* infections and outbreaks in humans are caused by unspecified environmental sources and the contamination of non-meat food items such as produce. (CDC 2007; Jain et al. 2019) This indicates that wild animals, including birds, have a place in the transmission cycle of *Salmonella* in humans. In fact, in 2021, a multistate human salmonellosis outbreak was traced to contact with wild birds experiencing a salmonellosis epornitic (CDC 2021). This relationship is bidirectional, as human activity can facilitate the transmission of *Salmonella* into wild bird populations. For example, the black-headed gull (*Larus ribibundus*), which has a *Salmonella* prevalence of around 2-30% (Palmgren et al. 2005; Čížek et al. 2007), has been detected shedding the same serotypes of *Salmonella* found in nearby human sewage outputs where they forage (Fricker 1984). Other studies have utilized the PulseNet database of human cases maintained by the CDC to confirm that the same serotypes or strains active in human populations in a given year are also found in urban-associated wild birds such as various gull species and the American white ibis (Dolejska et al. 2016; Hernandez et al. 2016; de Oliveira et al. 2018).

Additionally, as *Salmonella* is commonly transmitted through consumption of contaminated food or water, *Salmonella* in birds can be an indicator of environmental contamination and shifts in habitat use. Numerous studies on gull species, which are major *Salmonella* reservoirs due to their scavenging feeding ecology, have demonstrated how environmental characteristics and animal foraging habits are mirrored in the prevalence of *Salmonella* in their populations, such as one study on urban herring gulls (*Larus argentatus*) in which prevalence was higher in populations that foraged near sewage sites than those that foraged near landfills (Monaghan et al. 1985; Tizard 2004).

One study in Patagonia found a significantly higher *Salmonella* prevalence in American black vultures (C*oragyps atratus*) foraging at a landfill compared to those foraging in the natural steppe environment, despite other pathogens exhibiting no such difference (Plaza et al. 2019). These environmental effects on *Salmonella* prevalence in wild birds combine both ecological and public health interests.

Significance of Salmonella in Urban Birds

This focus on the effects of landscape and wildlife ecology on *Salmonella* is particularly relevant in the context of urban birds. With continuing human alteration to the landscape and the expansion of development, birds may congregate in increasingly limited areas, including urban zones. In these areas, landscape contamination with human waste and increasing bird density can exacerbate risk factors for *Salmonella* transmission. Similar to the studies on herring gulls, American black vultures and songbird species, the characteristics of urban foraging and urban-associated supplemental feeding sites commonly lead to the increased *Salmonella* prevalence of these populations (Monaghan et al. 1985; Kirkwood and Macgregor 1998; Camacho et al. 2016; Galbraith et al. 2017; Plaza et al. 2019; Martín-Maldonado et al. 2020). The contamination and density of urban foraging sites may also combine with urbanization-induced health changes caused by shifts in community, diet composition, and stress and lead to significant changes in *Salmonella* epidemiology in these areas (Ditchkoff, Saalfield, and Gibson 2006; Bradley and Altizer 2007; Murray et al. 2020). These changes could include shifts in wild bird mortality rates as well as emerging threats to public health.

The American White Ibis System

The American white ibis (*Eudocimus albus*) is a nomadic wading bird common across the Southeast. They are tactile foragers that use their long beaks to probe in shallow water (typically from 10-32 cm) or vegetation and locate their prey, often moving in loose flocks of varying size and species composition as they forage (Kushlan 1977a; Kushlan 1979; Binkley, Dorn, and Cook 2019). They commonly consume prey such as crayfish, insects, fish, and small amphibians, with diet composition dependent on their foraging habitat (Kushlan 1979; Kushlan, 1980). In the past 20 years, they have become common in urban parks, backyards and other spaces, foraging on terrestrial invertebrates and provisioned with anthropogenic food such as bread and other processed items. (Frederick et al. 1996; Hernandez et al., 2016).

Ibis tend to breed in large, mixed species rookeries during the spring and early summer, traditionally in large natural wetlands (Kushlan 1977b). In addition to populations of a number of other colony nesting aquatic bird species, these colonies can house as many as 60,000-100,000 ibis, which rely on large areas of land around the colony for foraging. As the need for prey is highest at this time, changes in the suitability of these foraging sites, such as changes in water level or prey availability, can cause shifts in breeding colony location and size (Kushlan 1977b; Frederick and Ogden 1997). Recently, ibis have begun breeding in smaller colonies found in urban areas, possibly driven by the consistent food source provided by human refuse and provisioning.

Salmonella in the American White Ibis

Salmonella dynamics have been well studied in ibis in order to understand whether *Salmonella* has negative effects on ibis populations, whether *Salmonella* in ibis poses a public health risk, and to determine whether and how the urban transition impacts the epidemiology of *Salmonella* in this species. Wild adult ibis have been found to be competent hosts for a high diversity of *Salmonella* serotypes and genotypes, including those found in humans, and evidence indicates that birds are frequently infected, recover, and then get reinfected with different *Salmonella* serotypes (Hernandez et al. 2016). This well-established host-pathogen relationship can then be examined more closely, comparing pathogen prevalence across age and urbanization gradients (Becker et al. 2018; Faust et al. 2018).

Adult American white ibis can carry *Salmonella* without developing clinical disease, shedding intermittently without known effects on their fitness (Tizard 2004; Hernandez et al. 2016). *Salmonella* sp. prevalence in ibis (average 26%) was found to increase as the proportion of wetland and herbaceous grassland decreased and open-developed land (e.g. golf courses, parks) increased (Hernandez et al. 2016). This indicates that developed areas may support higher pathogen loads than natural areas, which has been attributed to changes in feeding ecology and gut microbiota (Hernandez et al. 2016; Murray et al. 2020). Ibis have high foraging habitat fidelity (Kidd 2018) and thus urban ibis likely forage primarily within urban zones, where sites may be contaminated with sewage and the feces of other known *Salmonella* reservoirs that frequent beaches and parks within the ibis range, such as gull, duck, and goose species (Tizard 2004). In urban areas, limited foraging locations are often used by higher densities of individuals, further increasing the risk of transmission. Mathematical modeling of urban-foraging ibis *Salmonella* prevalence has indicated that sustained transmission in ibis populations is possible even without non-ibis sources of *Salmonella*, though models which include both within-species and cross-species transmission tend to be best fit to observed *Salmonella* trends in these populations (Becker et al. 2018). This indicates that proximity to shedding conspecifics within an urban-foraging ibis flock is an important transmission mechanism in this system, either through contact or environmental contamination, though other cohabitating species also contribute to the environmental pool of *Salmonella* that ibis are affected by.

The Significance of Salmonella in Ibis Nestlings

The epidemiology of *Salmonella* in nestlings has been understudied in previous work, but their growing association with urban habitats necessitates further research. Nestlings in urban breeding colonies may be experiencing a mix of age-specific physiological factors and environmental effects that could influence their *Salmonella* prevalence and the occurrence of clinical disease compared to natural rookeries.

Inherent Susceptibility of Nestlings to Salmonella

In one study of natural ibis breeding colonies, the prevalence of *Salmonella* was higher in nestlings (35%, N=72) than in adults (13%, N=261) (Hernandez et al. 2016). This indicates that, even in typical ibis breeding environments not experiencing urban effects, nestlings are more susceptible to infection, though no clinical disease was detected. This pattern of age-dependent prevalence holds in some wild birds and in the domestic chicken (*Gallus gallus domesticus*), the model species for *Salmonella* in birds (McCrea et al. 2006; Palmgren et al. 2006; Phalen et al. 2010). In some wading bird species, this increased prevalence of nestlings is accompanied by more common occurrence of disease, as is the case for the colony-nesting cattle egret (*Bubulcus ibis*). In these birds, nestlings have higher prevalence of *Salmonella* compared to adults (29-91%

nestlings, 0% adults) and infections were often associated with mild hepatitis and higher instances of chick mortality (Phalen et al. 2010).

In the review of bird mortalities from the USGS database from 1985 to 2004, 16% (N= 53) of lethal *Salmonella* outbreak events in colony nesting birds exclusively killed nestlings and juveniles. This apparent increase in prevalence of *Salmonella* and susceptibility to salmonellosis in young birds is likely caused in part by their immune naivety, with their acquired immune responses less capable of recognizing salmonellae than those of adults. Another important factor in young bird infection and disease susceptibility is their immature GI microbiome. Salmonellae must adhere to and inject themselves into the epithelium of GI organs, but in a mature bird they must compete with a huge number and diversity of commensal bacterial organisms to do so (Foley et al. 2013; Rogers, Tsolis, and Bäumler 2021). Nestlings have not yet acquired this diverse microflora and *Salmonella* spp may more readily enter their cells (Azcarete-Peril et al. 2018; Teyssier et al. 2018; Davidson et al. 2021; Rogers, Tsolis, and Bäumler 2021).

In ibis, though we know that risk factors such as these have led to higher prevalence of nestlings than adults, we do not know whether this commonly leads to disease or how these infections progress, as the Hernandez et al. study did not follow nestlings through the course of infection (Hernandez et al. 2016). The potential presence of clinical signs or mortality and the duration of shedding are of interest in evaluating how *Salmonella* affects these birds. Additionally, in ibis and other species, the timeline at which immunity develops in young birds is not well studied, so it is unclear whether the early infection of nestlings leads to decreased *Salmonella* susceptibility after recovery.

These details of the age-specific epidemiology of *Salmonella* in ibis are crucial for understanding this host-pathogen relationship.

<u>Habitat-Dependent Factors Influencing Salmonella Dynamics in Nestlings</u>

Nestling ibis may also experience habitat effects that alter rates of *Salmonella* transmission, compounded with their inherent susceptibility to infection and disease from this pathogen. The characteristics of the colony-nesting environment create many opportunities for pathogen transmission, with many individuals and species gathered for weeks at a time, leading to potential contact with infected birds and possible surface and water contamination by feces (Brown and Brown 2004; Sovada et al. 2008; Iverson et al. 2016). Natural ibis breeding colonies tend to be relatively dense and ibis cohabitate with many other aquatic colonial species, some of which, such as the little blue heron (*Egretta caerulea*), black crowned night heron (*Nycticorax nycticorax*), and double crested cormorant (*Phalacrocorax auritus*) are known to shed *Salmonella* and may develop clinical disease (Locke et al. 1974; Kushlan 1977a; Clavijo, Robinson, and Lopez 2001; Dobbin et al. 2005; Hall and Saito 2008). These potential transmission routes may be even more active in urban colonies where the limited land area could lead to denser gatherings of birds within the colony. Rookeries in urban zones are more likely to harbor waterfowl species such as mallards (*Anas platyrhynchos*), another *Salmonella* host species that could potentially contaminate the area around these vulnerable nestlings (Tizard 2004; Krawiec et al. 2015). Additionally, if we assume that the parents of urban ibis nestlings are urban foragers, they may have elevated *Salmonella* prevalence due to their foraging habits and diet and thus expose their offspring to the same risk factors through contact and diet provisioning. The reduced gut microbiome diversity of urban-foraging adult ibis has been associated with the elevated *Salmonella* prevalence of these birds, so the consumption of this same diet by nestlings may stunt the maturation of their own commensal microbial community (Murray et al. 2020). Finally, urban breeding colonies subject nestlings to urban-associated stressors such as noise and pollution, with potential health effects that could further alter the outcome of nestling exposure to *Salmonella* (Ditchkoff, Saalfield, and Gibson 2006; Bradley and Altizer 2007). As such, studying the prevalence and progression of *Salmonella* infection in free-living ibis nestlings in both natural and urban habitats may illuminate the way this pathogen interacts with wild colony-nesting young birds in addition to the ways in which the urban transition can affect this relationship.

Hypotheses and Objectives

To understand the *Salmonella* dynamics of ibis nestlings, we conducted various studies of nestlings in captive, urban, and natural settings. In 2017, we monitored a captive colony of wild-caught nestlings for *Salmonella* for their first six months of life in order to detect the prevalence, shedding periods, and reinfection dynamics of maturing ibis. We then experimentally infected those nestlings as juveniles to determine if susceptibility to *Salmonella* infections early in life provided immunity later. We predicted a high prevalence in the early weeks of captivity that would gradually decline, with some occurrences of clinical disease or mortality among the young birds.

We also sampled free-living nestlings in both urban (2020 and 2021 breeding seasons) and natural (2021 breeding season) colonies weekly for the first two to three weeks of life to identify *Salmonella* prevalence and serotype diversity among young nestlings in the wild and detect any changes that occur in novel urban breeding colonies.

Among these free-living nestlings, we also predicted a high prevalence of *Salmonella* shedding causing disease or mortality, with higher prevalence in the urban colonies than in the natural colonies.

This work fills in gaps in knowledge about the *Salmonella* dynamics in young ibis, producing the most complete age-specific findings on *Salmonella* epidemiology in an avian species besides the domestic chicken.

Methods

Salmonella Dynamics of a Captive Colony of White Ibis Nestlings

Nestling Capture, Care, and Monitoring

As part of a larger project, 24 American white ibis nestlings from a wetland rookery in Broward County, FL were hand-captured at an average age of 14 days in April 2017. They were banded for identification and transported in carriers via vehicle to the University of Georgia's Poultry Diagnostic Research Center in Athens, GA for captive rearing. They were housed as a single group in a modified chicken house for the first stage of their rearing. They were fed a piscivore gruel (Emeraid) and had a strictly controlled room temperature. The 3.9 m x 5.9 m pen had cement floors covered in artificial grass matting, a variety of ground and hanging perches, a wading pool, browse, and full-spectrum lights (ZooMed Avian Sun 5.0). After three weeks of captivity, birds were split into two groups by age, where birds that required syringe feeding were housed in one pen and older birds able to eat from food trays in another. During this stage, birds were sexed using standard molecular techniques (Fridolfsson & Ellegren 1999) and fed a solid diet. After three months of captivity, the birds were again randomly selected into two equal sized groups while maintaining an even sex ratio (N= 10 per pen, 5 males and

5 females). The room temperature was consistent with natural Florida temperature for the season, and their diet was altered to meet the nutritional needs of the growing ibis. The two groups were fed different diets beginning at captive day 113, with one group randomly assigned to receive a diet consistent with urban foraging ibis (reduced protein, higher simple carbohydrates).

In the first 5 weeks after capture, birds were handled daily and body weight and morphometric measurements were collected; thereafter, they transitioned to being handled on alternating days from days 37 to 58 post-capture, and after two months in captivity, birds were handled only once per week. Throughout the entire captive period, fecal samples were collected approximately once per week, though not every bird was sampled each week after the initial weeks of captivity. Approximately 1 g of the fecal sample was submerged in 10 ml of dulcitol selenite broth, stored at room temperature, and brought to the University of Georgia Environmental Health Department for analysis the same day (See '*Salmonella* identification and analysis' for detailed methods).

Salmonella Experimental Infection Trial

At approximately six months of age, birds were moved to a BSL-2 facility and the housing groups were rearranged such that there were equal ratios of birds from each diet treatment in each of the two new houses to create a blocking effect. On the day of inoculation (DP0), birds were inoculated with a 10^6 *S. typhimurium* suspension orally. Fecal samples were then collected daily for 14 days. All fecal samples were embedded in 10 ml of dulcitol selenite broth, stored at room temperature, and transported to the University of Georgia Environmental Health Department for analysis the same day (See '*Salmonella* identification and analysis' for detailed methods).

All animal care procedures were approved by the University of Georgia Institutional Animal Care and Use Committee (UGIACUC: Animal Use Permit A201609-012-A1). Nestlings were collected under Florida scientific collection permit LSSC-11-00119G, a federal collection permit MB779238-2 through the Southeastern Cooperative Wildlife Disease Study at the University of Georgia and transported under Georgia Department of Natural Resources Import Permit 1000529276.

Salmonella Dynamics of Free-Living White Ibis Nestlings

<u>*Study Sites*</u>

We collected fecal samples from nestlings in both urban and natural rookeries weekly during their first weeks of life. Maps and photos of all rookery sites are shown in Figures 1-4. The urban site, sampled during the March through July breeding seasons in both 2020 and 2021, contained three adjacent mixed-species rookery tree islands in the PGA National Resort and Spa, a gated community in Palm Beach Gardens, FL (Figures 1 and 2). These islands were located in a single artificial body of water between a residential community and a golf course. The islands, called A, B, and C, were 542 m^2, 478 m^2, and 1312 m^2 in area, respectively, and composed of palm trees (approximately 10 m tall), Ficus, Brazilian pepper, and other trees and shrubs. The islands had dense canopies with relatively open interiors (Figure 3A-D). Other aquatic bird species utilizing the islands included little blue herons, great egrets (*Ardea alba*), and double-breasted cormorants (*Phalacrocorax auratus*), as well as smaller groups of Egyptian geese (*Alopochen aegyptiaca*), anhinga (*Anhinga anhinga*), glossy ibis (*Plegadis falcinellus*), snowy egrets (*Egretta thula*), black crowned night herons, tricolored herons (*Egretta tricolor*), and great blue herons (*Ardea herodias*). Potential predator species such as

American crows (*Corvus brachyrhynchos*), blue jays (*Cyanocitta cristata*), and American alligators (*Alligator mississippiensis*) were observed in and around the islands. Two island rookeries in natural wetlands within the Everglades Water Conservation Area 3A and Frances S. Taylor Wildlife Management Area were also sampled during the 2021 breeding season (Figure 1). These sites, called Alley North and Hidden, have been active rookeries for wading birds in the Everglades from at least three decades, and were accessed by airboat and then on foot. Hidden is approximately 50,000 m^2 in area and the vegetation is dominated by cypress trees and cocoplums (Figure 4A-B). It was dominated by white ibis during the 2021 breeding season, though some great egrets and small heron species were present. Alley North, approximately 50,000 m^2 in area and often the rookery with the largest population in this region, is comprised primarily of willow and cypress trees surrounded by cattails and sawgrass (Figure 4C). Many other wading bird species, in addition to ibis, utilized this island in 2021, including great egrets, roseate spoonbills (*Platalea ajaja*), glossy ibis, tricolor herons, and more.

<u>Sampling Methods</u>

Nestlings at each site were sampled repeatedly over 1-4 weeks in order to track their *Salmonella* status and other health-related metrics. At the urban rookery, each of the three islands was visited at least once per week. Personnel entered the island at approximately 6:30 am and exited between two to five hours later. All nests that were within reach or that could be checked with a mirror pole were numbered and monitored weekly (Figure 2, Figure 3D), in order to begin nestling sampling as soon after hatching as possible. A similar procedure was followed at the natural sites, but sampling time was constrained to one hour to avoid adult disturbance, and nests were not monitored prior to nestling hatching.

Once hatched, the oldest nestlings from all accessible nests were captured once per week by hand or using a wire-loop snare pole for as many weeks as capture was possible. All sampled nestlings were banded to ensure consistency across weeks, with plastic bands appropriate for bird leg size changed weekly until a permanent band could be fitted. Nestling age range during sampling was 1 day to 25 days old. Body weight and standard avian morphometric measurements (culmen length, tarsus length, tarsus width, and wing chord) were collected for subsequent assessments of growth and body condition. To detect *Salmonella* shedding, a fecal sample was collected from fecal piles when birds defecated during the sampling process and from fecal swabs when they did not. Approximately 1 g of the fecal sample was inoculated in 10 ml of dulcitol selenite broth, stored at room temperature, and shipped to the Lipp Lab in the University of Georgia Environmental Health Department in Athens, GA within 2-4 days of collection. Rookery access was granted through North Palm Beach County Improvement District (PER 11-464). Scientific collecting permits were granted through the U.S. Fish and Wildlife Service (MB53675D-0) and Florida Fish and Wildlife Conservation Commission (LSSC-11-00119H). All animal procedures were reviewed and approved by the University of Georgia's Institutional Animal Care and Use Committee (AUP A2019-10-009-Y1-A0).

In the event dead or moribund birds which were later euthanized, were found on the islands, carcasses were collected and frozen at 4ºC and shipped or transported to SCWDS for analysis.

Salmonella Identification and Analysis

Salmonella isolation was completed as previously optimized (Odumeru & Leon-Velarde 2012; Lee et al. 2015). Upon receipt of the samples, a 100 µL aliquot of the enrichment media was inoculated into Rappaport Vassiliadis broth and incubated at 43ºC with 100rpms of shaking agitation. Using a sterile loop, 10µL of this sample was then streaked onto XLT-4 agar selective plates and incubated at 37ºC overnight. Presumptive *Salmonella* colonies, which appear black, were then transferred as patches onto ChromAgar *Salmonella* Plus Agar selective media to confirm *Salmonella* identification, with positive patches appearing mauve in color after incubation. *Salmonella* isolates from captive individuals and wild nestlings during the 2020 breeding season were sent to the National Veterinary Services Laboratory (NVSL) in Ames, Iowa for serotyping. Briefly, serotype analysis is completed by mixing the isolate bacterial cells with antibodies and using the combination of agglutination patterns to identify the antigen variants of that isolate.

Data Analysis

Statistical analysis and data representation were completed using R version 4.0.3 and the ‘dplyr’, ‘ggplot2’, ‘lme5, and ‘lmtest’ packages. A generalized linear mixed model (GLMM) was fit using habitat type, age in weeks, and an estimate of body condition as fixed effect variables and the bird ID and number of captures as random effects, to account for the both the presence of and variation in repeated sampling of individuals. Other models were considered using stepwise model selection regression with Akaike Information Criterion (AIC) and variables were ultimately selected based on AIC and relevance to the experimental design, with further validation later using likelihood ratio tests. Age in weeks was defined as follows: a bird between 0-6 days old was marked as 0 weeks, 7-13 days as 1 week, and 14 days or older as 2 weeks. The body condition estimate was calculated using methods previously validated in ibis (Herring et al. 2014; Murray et al. 2020), by regressing tarsus length (determined to be the structural measure most strongly correlated to mass) to weight, and then calculating the residuals as a percentage of the predicted mass. This range of residuals was then divided into five equal ranges and body condition was assigned along this scale.

Results

Captive Nestling Prevalence and Experimental Infection

Fifty-eight percent (N=24) of the wild-caught nestlings were shedding *Salmonella* at the time of capture, with only two serotypes (*S.* Javiana and *S.* Poona) isolated. During captivity, prevalence increased and remained high (~67%) until approximately 7 weeks of age (Figure 2.5). At this point, prevalence declined to zero, except for a few isolations at week 10 and 17, after which it remained at zero for 8 weeks prior to the experimental infection trial. The length of shedding between individuals varied widely and, at times, some birds began shedding again after testing negative. The timeline of shedding for ten selected individuals is shown in Figure 6 to illustrate the variation between nestlings.

After experimental inoculation in the morning, we confirmed the passage of the inoculant by collecting feces the same afternoon and detected *Salmonella* in 35% of all birds (N=20). Subsequently, three birds shed *Salmonella* on DPI-1, one of which developed clinical disease (fluffing of feathers, hyperthermia, and anorexia) from which it recovered within 24 hours. Another bird shed on DPI-5. No birds shed *Salmonella* for more than one day.

The Salmonella Prevalence of Wild White Ibis Nestlings

A total of 117 nestlings were sampled across all sites and years, with a mode of 3 captures per individual (Table 2.1). The average number of captures per bird was lower in the natural rookeries (1.74, N= 39) than the urban rookeries (2.68, N=78) due to access differences between the sites.

Prevalence of Samples

We found relatively high prevalence of *Salmonella* among young free-living nestlings, with an average prevalence at the sample level of 43% (N=269), including all repeated measures of the same individuals, taken across both sites and years (Table 2.1). The prevalence of urban samples (52%, N=209) was higher than the prevalence of samples from natural colonies (13%, n=60), and this difference was statistically significant (F-statistic 16.3, $p<0.001$). Within a habitat type, the individual site (A, B, C, Alley North, or Hidden) did not yield statistically significant differences in the prevalence of samples (natural: F-statistic= 0.28, p-value= 0.60; urban: F-statistic= 0.50, p-value= 0.61). When examined by bird age in weeks, the prevalence of samples tended to decrease with age, though this difference was not statistically significant (F-statistic 1.8, p=0.18) between any age group, and there was variety in trend by site and year (Figure 7). In the urban rookery, prevalence of 2020 samples was slightly lower (49%, N= 94) than prevalence of 2021 samples (55%, N= 115), but this difference was not statistically significant (F-statistic= 1.7, p= 0.19) (Figure 7). The prevalence of fecal swab samples (35%; N=55) was lower than the prevalence of fecal samples (49%; N=214), a non-statistically significant difference (F-statistic= 1.5, p= 0.22) but one that has been confirmed to be non-random in similar studies (Martín-Maldonado et al. 2019).

Individual Salmonella Status Over Time and Prevalence by Individual

In total, 60% of individuals (N=117) tested positive at least once during the sampling season. A summary of their *Salmonella* status over time is on Table 2.2 and overall prevalence data from individuals at each site and year is on Table 2.3. Birds in the urban rookery were more likely to shed *Salmonella* than those in the natural rookery (likelihood ratio test 25.304, $p<0.0001$). At the urban rookery in 2020, 81% of sampled birds (N=36) tested positive at least once, with 14 individuals positive more than once and 10 individuals (34% of all positive birds) shedding during multiple consecutive weeks, yielding a total of 45 *Salmonella* isolates. In this group, 17 *Salmonella* serotypes were isolated (Table 4), with *S. Reading* as the most common serotype, identified from 16% of isolates. In 2021, 86% of birds (N=42) from the urban rookery tested positive at least once during sampling and 47% of these tested positive for multiple consecutive weeks, plus one bird testing positive twice but not consecutively. Only 18% of birds (N=39) from the natural area rookeries tested positive, with a single bird shedding for more than one week. No mortality events were associated with *Salmonella* infection during 2020 and no evidence of clinical disease was observed during either year.

In addition to these reported prevalence data, the significance of factors affecting nestling *Salmonella* status was evaluated using a generalized linear mixed model (Table 2.5). In the final model, habitat type had the largest marginal effect (likelihood ratio test 25.304, $p<0.0001$) and scaled mass body condition estimate and bird age in weeks were the next most useful variables, with likelihood of *Salmonella* shedding decreasing with increasing body condition (likelihood ratio test 6.2, $p<0.05$) and increasing age (likelihood ratio test 2.8, $p <0.1$) when repeated measures and habitat type were accounted for, though age and body condition were strongly correlated to each other. By backwards model selection, the site ID (likelihood ratio test 1.2264, p=0.9423) and bird ectoparasite score (likelihood ratio test 0.2243, p=0.6358) were not significant predictors of *Salmonella* status, and the scaled body condition measurement (likelihood ratio test 6.0325, p= 0.01404) was a better predictor than the field-estimated body condition (likelihood ratio test 0.78, p= 0.377).

Discussion

Captive Nestling Prevalence and Experimental Infection

In the captive colony, we found that *Salmonella* prevalence was high from the time of capture until about 7 weeks of age when it declined rapidly. The monitoring of the captive colony provides a detailed timeline of *Salmonella* shedding and reinfection among 2-week to 6-month-old ibis consistently exposed to the bacteria due to captive conditions. At the time of capture from the wild at approximately two weeks of age, prevalence was around 54% and the subsequent rise then decline in the prevalence of individuals shedding *Salmonella* shows how, even with young nestlings in dense living conditions, *Salmonella* shedding eventually runs its course and clinical disease is rare, despite some individuals shedding *Salmonella* for over a month (Figure 2.4).

This long shedding duration is consistent with studies of hatchling domestic chickens. One study of chicks experimentally infected on the day of hatching (N=144) found that *Salmonella* colonized the liver and spleen during the first weeks of infection but that bacterial cell counts were drastically reduced by 4 weeks post-inoculation and reached 0 by 8 weeks (Gast and Holt 1998). Cecal colonization and fecal shedding in these birds, however, persisted in a large proportion of birds for at least 16 and 24 weeks, respectively. The particularly long duration of intestinal *Salmonella* infection in this study is likely related to the relationship between the host and the dose and strain inoculated, but demonstrates general patterns in chicken infection dynamics: chick susceptibility to non-GI organ colonization and salmonellosis is reduced after the first week of life as the immune system matures, and that chicks can shed *Salmonella* in feces intermittently for long periods without showing clinical signs, as we observed in ibis nestlings (Milner and Shaffer 1952, Sadler et al. 1969). Nestlings in our study, however, were captured between 1 and 2 weeks of age, beyond some of the benchmark ages at which the chicken *Salmonella* resistance mechanisms improve (increased heterophil activity, humoral immune response, etc.) (Wells et al. 1998; Holt et al. 1999). This left open questions as to whether *Salmonella* affected ibis health and survival during the first days post-hatching.

After several months without *Salmonella* detection, experimentally infected young juvenile ibis may be resistant to re-infection, with only 4 birds shedding in response to the inoculation, none for more than one day and only one with clinical signs. These results indicate that exposure to *Salmonella* sp. at a young age may provide a degree of immunity to older nestlings and juveniles; however, further research sampling adult individuals repeatedly would be needed to explore this further. While *Salmonella* infections do not typically lead to the development of independently effective humoral immunity due to the rapid invasion of host cells by the bacteria, some acquired cellular immunity in the GI tract can be developed after infection, decreasing later susceptibility (Mastroeni 2002; Wigley 2013; MacLennon 2014). This process is well studied in chickens for the development of *Salmonella* vaccines. Studies have indicated that, in vaccinated animals, long-term protection relies on a combination of antigen-specific responses including antibody, T-cell, and cytokine activity, but that at least partial protection can be granted by other methods of immune activation, including persistent infection, as we observed in nestling ibis (Mastroeni and Ménager 2003; Wigley 2013).

The Salmonella Prevalence of Wild White Ibis Nestlings

Free-living nestlings were sampled from the time of hatching until approximately 3 weeks of age, complementing the ages of the nestlings sampled in the captive colony. These birds shed at a relatively high prevalence of *Salmonella* even just a few days after hatching, but still did not develop clinical disease or mortality, even in the urban colony where prevalence was particularly high. Their *Salmonella* prevalence varied with age by site and year, initially decreasing between the first and second week of life in the natural colony and in the urban colony 2020 but increasing over the same period in the urban colony in 2021. Within the urban colony, total prevalence of samples was relatively consistent across years (~49% in 2020 and 55% in 2021).

For both total samples collected and the individual nestling *Salmonella* status over time, *Salmonella* prevalence was lower in the natural rookeries than the urban rookery. Furthermore, GLM models demonstrated that the habitat type was the most significant predictor of *Salmonella* status for individual birds, with repeated measures and the variable capture numbers accounted for as random effect variables. This is consistent with predictions, based largely on the observed prevalence of ibis adults in urban areas (Hernandez et al. 2016). Ibis nestlings in urban colonies are more likely to have a *Salmonella*-positive parent due to changes in pathogen prevalence caused by urban foraging and the urban ibis diet (Hernandez et al. 2016; Kidd-Weaver 2020; Murray et al.

2020). This exposure via their parents, combined with the high density of ibis nests within the urban breeding colony (often 2-5 nests in a single tree) and the likely high shedding rate of immunologically vulnerable nestlings, leaves ample opportunity for transmission, especially when combined with other factors such as the occurrence of more *Salmonella* reservoir species in urban colonies and the changing health conditions related to diet and stress (Ditchkoff, Saalfield, and Gibson 2006; Bradley and Altizer 2007).

This study is particularly powerful, however, as not many others have been able to directly compare nestling prevalence in separate rookeries through a full breeding season. Other studies establishing pathogen prevalence differences of birds in urban versus natural conditions often rely on single visitations to nests or foraging sites (Monaghan et al. 1985; Rosenfield et al. 2002; Plaza et al. 2019; Martín-Maldonado et al. 2020), preventing the analysis of some inter- and intra-individual data relevant to pathogen epidemiology. The design of this study, with weekly sampling of tracked birds and continued sampling through the season, allowed for high resolution and confidence in our cross-habitat comparisons, while the duplicate years of study in the urban rookery provide added security in the validity of the detected prevalence of urban nestlings, a previously unstudied novel subpopulation of ibis. Additionally, when paired with the captive colony data, these findings establish that ibis, even young nestlings experiencing persistent *Salmonella* infections, do not commonly die from *Salmonella*.

GLMM analysis showed that, in addition to habitat type, *Salmonella* status was also associated with bird body condition and age in weeks. Increasing body condition was associated with decreasing likelihood of *Salmonella* shedding after accounting for habitat type. These effects were crucial to parse from each other due to the association of higher body condition with urban nestlings, as if often the case in urban adults compared to those in natural areas (Hernandez et al. 2016). The association between lower body condition and increased *Salmonella* likelihood is consistent with findings of poor body condition in individuals of other species that have died from *Salmonella*, though there are few studies on the varying susceptibility of birds to infection based on body condition (Mikaelian et al. 1997; Benskin et al. 2009; Lawson et al. 2010). Age was a slightly significant predictor of *Salmonella* status of nestlings, with increasing age in weeks slightly decreasing the likelihood of *Salmonella* positivity. This is likely related to the gradually-maturing GI microbiome and inherent immune response of nestlings, decreasing their susceptibility to infection. This is an established pattern in the study of *Salmonella* in chickens and thus was included in the model despite its relative underperformance in the GLMM (Sadler 1969; Davidson et al. 2021).

Due to the reduced viability of fecal swabs versus fecal samples, prevalence approximations in this study may slightly underestimate the true prevalence of nestlings sampled. A similar study of *Salmonella* prevalence in Bonelli's eagle nestlings (*Aquila fasciata*) confirmed that fecal swabs have a lower *Salmonella* detection efficiency than fecal samples, consistent with our results and previous experience (Martín-Maldonado et al. 2019; Hernandez pers comm).

Salmonella Serotypes

In the captive colony, *S.* Javiana was the most common serotype found in the nestlings. Although *S.* Poona was isolated from one bird soon after capture, it was dominated by *S.* Javiana. In the wild nestlings, *S.* Reading was the most common serotype isolated, though a few other serotypes persisted throughout the breeding season and 17 serotypes were isolated in total, indicating that nestling infections came from a variety of sources rather than a single outbreak. In ibis, the *Salmonella* serotypes shed tend to reflect their habitat and diet (Hernandez et al., 2016). *S*. Javiana, *S*. Poona, and *S*. Reading are all among the top 32 serotypes of concern for human health, and in Florida, *S*. Javiana was the 3rd most common *Salmonella* serotype isolated from humans in 2017-18, and *S.* Poona was the 13th most common (Li et al. 2021). *S*. Reading was not in the top 20 serotypes isolated from humans in Florida during this time period, though several other serotypes isolated from nestlings in 2020 were on this list, including *S.* Sandiego and *S*. Braenderup, which were the 4th and 5th most common serotypes (Li et al. 2021). In the CDC's *Atlas of Salmonella in the United States*, the most common non-human sources of *S.* Javiana identification were horses and turkeys. For *S.* Poona, non-human isolates typically came from reptiles. Turkey were again the most common source of *S.* Reading isolates. These apparent sources, however, are strongly biased by sampling effort in particular animal groups and thus do not conclusively identify the ultimate source of these serotypes. In recent years, a number of the serotypes isolated in this study were also isolated from other aquatic birds, including *S.* Typhimurium from great egrets in Brazil in 2014 (de Oliviera et al. 2018) from a variety of duck and wading bird species in Argentina between 2014 and 2016 (Rodríguez et al. 2018). Additionally, *S.* Typhimurium and *S.* Newport were isolated from cattle egrets (Silva et al. 2018) and neotropical cormorants (*Nannopterum brasilianus*) (Cardoso et al. 2021) in Brazil in 2008 and 2015.

Conclusions, Limitations, and Future Directions

Overall, these results indicate that, while *Salmonella* infection is common among nestlings and shedding can last for multiple weeks, clinical disease is rare and these early infections may lead to decreased susceptibility in later life stages, though only one serotype was used to challenge nestlings and the mechanism and persistence of this protection is unknown. Additionally, there were no captive nestlings which had never shed *Salmonella* at the time of experimental inoculation, so the attribution of early infections to the resistance shown is based on observed free-living juvenile prevalence data and established trends in acquired *Salmonella* immunity in chickens (Mastroeni and Ménager 2003; Wigley 2013; Hernandez et al. 2016). The consistency between ibis nestling epidemiology in captive and free-living conditions confirms the validity of major conclusions drawn from the captive colony data, such as the lack of clinical disease and the duration of the shedding period.

The increased prevalence of nestlings in the urban colony matches our predictions based on previous work in adults and consideration of the urban rookery conditions, and the associations with age and body condition also hold with general assumptions discussed above about *Salmonella* susceptibility. The variation in sampling structure (travel time to and from site, amount of time spent sampling, etc.) may have had some influence on detected *Salmonella* prevalence due to the differing constraints of access to the natural versus urban rookeries, though sample collection and storage were as consistent as field conditions would allow. Against our predictions, we found no evidence of disease or mortality among our sampled nestlings related to this high prevalence, however it is possible that some chick deaths, particularly of untagged birds, went unnoticed and could thus not be analyzed for association with *Salmonella*, as rookeries were not visited every day and were frequented by potential scavengers. Either way, the identified shift in pathogen dynamics in urban areas may make ibis nestlings vulnerable to other health problems associated with urban zones, such as stress and contaminants. When combined with the information from the captive colony and previous work on the American white ibis, these results provide the most complete age-specific accounting of *Salmonella* in a wild avian species.

Future work should include sampling of the ibis adults, environment, and community around nestlings to better understand the *Salmonella* inputs and outputs active in these colonies, particularly in urban environments. It is possible that these colonies, as they become more common, may pose a risk of spillover transmission to humans through the contamination of water around the colony or *Salmonella* transmission to species that may later come into contact with humans or pets. The proximity of the PGA rookery to human homes could facilitate visitation by, and *Salmonella* transmission to, birds like rock pigeons (*Columba livia*) and songbirds, increasing pathogen prevalence in these human-associated species and making spillover more likely. Conversely, if the observed protection against infection in young juveniles attributed to early *Salmonella* infection is persistent, the establishment of urban colonies with high prevalence of nestlings could reduce the prevalence of juveniles. These juveniles are more likely to come into contact with humans than nestlings are, and thus this shift in prevalence towards younger age groups could reduce the amount of *Salmonella*-positive ibis which humans encounter. Finally, in light of the increased *Salmonella* prevalence in these urban colonies, further study into whether *Salmonella* infection has sublethal or cumulative effects on nestling ibis could be valuable in understanding the changes being brought to their populations by this shift.

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Tables and Figures

Table 2.1: Fecal samples collected from nestlings for *Salmonella* isolation from American white ibis (*Eudocimus albus*) in two rookery habitats in South Florida in the 2020 and 2021 breeding seasons. Nestlings were sampled repeatedly up until approximately 3 weeks of age, and sample prevalence by age group is shown in the subgroups of each major column. Isolates obtained refer to samples that were confirmed to be *Salmonella* through both XLT-4 and ChromAgar analyses.

Year	Habitat Type	Site	Samples Collected				Isolates Obtained				Prevalence			
			Total	*0-6 days*	*7-13 days*	*14+ days*	*Total*	*0-6 days*	*7-13 days*	*14+ days*	*Total*	*0-6 days*	*7-13 days*	*14+ days*
2020	Urban	A	46	15	12	19	24	9	8	7	0.522	0.600	0.667	0.368
		B	41	16	14	11	20	9	3	8	0.488	0.563	0.214	0.727
		C	7	2	4	1	2	1	1	0	0.286	0.500	0.250	0.000
		Total	*94*	*33*	*30*	*31*	*46*	*19*	*12*	*15*	*0.489*	*0.576*	*0.400*	*0.484*
2021	Urban	A	38	13	12	13	23	6	8	9	0.605	0.462	0.667	0.692
		B	20	7	4	9	9	3	2	4	0.450	0.429	0.500	0.444
		C	57	19	17	21	31	10	13	7	0.544	0.526	0.765	0.333
		Total	*115*	*39*	*33*	*43*	*63*	*19*	*23*	*20*	*0.548*	*0.487*	*0.697*	*0.465*
	Natural	Hidden	40	10	13	17	6	2	2	2	0.150	0.200	0.154	0.118
		Alley North	20	0	14	6	2	n/a	2	0	0.100	n/a	0.143	0.000
		Total	*60*	*10*	*27*	*23*	*8*	*2*	*4*	*2*	0.133	0.200	0.148	0.087

Table 2.2: American white ibis (*Eudocimus albus*) nestlings sampled in urban and natural rookeries in South Florida during the 2020 and 2021 breeding seasons. Nestlings were sampled repeatedly from the time of hatching until capture was no longer possible, approximately 3 weeks. Individuals that tested positive for *Salmonella* spp., by XLT-4 and ChromAgar analyses of fecal samples and fecal swabs, at least once during sampling (N Positive) and the calculated prevalence within each site, habitat type, and breeding season are shown.

Year	Habitat Type	Site	Birds Sampled	N Positive	Prevalence
2020	Urban	A	15	13	0.867
		B	17	14	0.824
		C	4	2	0.500
		Total	*36*	*29*	*0.806*
2021	Urban	A	15	13	0.867
		B	7	7	1.000
		C	20	16	0.800
		Total	*42*	*36*	*0.857*
	Natural	Hidden	20	4	0.200
		Alley North	16	3	0.188
		Total	*36*	*7*	*0.194*

Table 2.3: *Salmonella* status (shedding (+) or not shedding (-), as detected by XLT-4 and ChromAgar analyses of fecal samples and fecal swabs) of American white ibis (*Eudocimus albus*) nestlings sampled in urban and natural rookeries in South Florida during the 2020 and 2021 breeding season. Birds were sampled repeatedly from the time of hatching until capture was no longer possible, approximately 3 weeks. Age groups where individuals were not sampled are marked with 'n/a/'. Birds which tested positive at any point during the given age range are marked (+) here, even if other samples within the same age range were negative.

Year	Habitat Type	Bird	*Salmonella* Status		
			0-6 days	*7-13 days*	*14+ days*
2020	Urban	1	-	-	+
		2	+	-	+
		3	+	-	n/a
		4	+	n/a	n/a
		5	+	-	+
		6	-	-	n/a
		7	-	+	-
		8	-	n/a	n/a
		9	+	-	-
		10	+	+	+
		11	+	n/a	-
		12	-	+	-
		13	+	n/a	n/a
		14	-	-	-
		15	+	+	-
		16	n/a	+	+
		17	+	-	+
		18	+	+	-
		19	-	+	+
		20	+	+	-
		21	-	-	-
		22	n/a	-	n/a

		23	n/a	-	n/a
		24	+	n/a	n/a
		25	+	+	n/a
		26	+	-	n/a
		27	-	n/a	n/a
		28	-	+	-
		29	+	+	+
		30	-	-	+
		31	-	-	n/a
		32	-	-	+
		33	+	+	n/a
		34	+	-	+
		35	n/a	-	+
		36	+	-	n/a
2021	Urban	101	-	-	+
		102	-	-	-
		103	-	-	-
		104	-	-	+
		105	-	+	+
		106	+	-	n/a
		107	-	+	+
		108	-	n/a	n/a
		109	-	+	-
		110	+	n/a	n/a
		111	-	n/a	n/a
		112	-	+	+
		113	-	+	n/a
		114	-	n/a	n/a
		115	-	+	+
		116	+	+	-
		117	+	+	+
		118	+	+	+
		119	-	+	-
		120	-	+	+
		121	+	n/a	n/a
		122	-	+	-
		123	+	-	-
		124	+	+	n/a
		125	+	n/a	n/a
		126	-	n/a	+
		127	+	+	+

		128	+	+	+
		129	+	-	-
		130	+	+	-
		131	+	+	-
		132	+	+	+
		133	+	+	+
		134	+	-	+
		135	-	+	-
		136	-	-	+
		136B	n/a	-	n/a
		137	n/a	+	+
		138	+	+	+
		139	+	-	-
		140	n/a	-	+
		141	+	n/a	n/a
	Natural	E01	n/a	-	-
		E02	n/a	n/a	n/a
		E03	n/a	n/a	n/a
		E04	n/a	n/a	n/a
		E05	n/a	-	-
		E07	n/a	-	-
		E08	n/a	+	-
		E09	-	+	+
		E10	-	-	-
		E11	-	-	-
		E12	-	-	n/a
		E13	-	-	-
		E14	n/a	n/a	-
		E15	n/a	n/a	-
		E16	n/a	-	n/a
		E17	n/a	-	n/a
		E18	n/a	-	n/a
		E19	n/a	-	n/a
		E20	n/a	n/a	-
		E21	n/a	-	n/a
		E22	n/a	-	n/a
		E23	n/a	-	n/a
		E24	n/a	+	n/a
		E25	n/a	-	n/a
		E26	n/a	-	n/a
		E27	n/a	-	n/a

		E28	n/a	-	-
		E29	n/a	-	-
		E30	n/a	-	n/a
		E31	-	+	-
		E32	-	-	+
		E33	+	-	-
		E34	+	-	n/a
		E35	-	n/a	n/a
		E36	n/a	n/a	-
		E37	n/a	n/a	-
		E38	n/a	n/a	-
		E39	n/a	n/a	-
		E40	n/a	n/a	-

Table 2.4: *Salmonella* serotypes isolated from fecal samples from American white ibis (*Eudocimus albus*) nestlings in an urban rookery in South Florida during the 2020 breeding season. Nestlings were sampled repeatedly from the time of hatching until approximately 3 weeks of age. All samples (N=94) were analyzed with XLT-4 and ChromAgar processes and isolates (N=45) were shipped to the National Veterinary Services Laboratory in Ames, Iowa for serotyping.

***Salmonella* Serotypes from 2020 Urban Rookery Nestling Isolates**	
Serotype	**Total Isolates**
Reading	7
Paratyphi_B_var._L-tartrate+	6
Sandiego	5
Baildon	4
Braenderup	3
Agona	3
Typhimurium	3
IV 50:z4.z23:-	2
Florida	2
Rubislaw	2
Manhattan	1
Johannesburg	1
Thompson	1
Bareilley	1
Saintpaul	1
Newport	1
Chester	1
IV_44:z4.z23	1

Table 2.4: Summary of a generalized linear mixed model (GLMM) (RStudio) evaluating factors which influence the *Salmonella* status (shedding or not shedding, as determined by XLT-4 and ChromAgar analyses) of American white ibis (*Eudocimus albus*) nestlings from urban and natural area rookeries in South Florida sampled over time during the 2020 and 2021 breeding season. Model was selected using Akaike Information Criterion (AIC) and includes the habitat type, bird body condition, and age as fixe effects models, and the bird ID and number of captures as random effects to control for repeated but variable sampling over time.

Fixed Effects	Description	Coefficient	p-value
Habitat	Effect of urban habitat	1.93	4.01E-06
Scaled Body Condition	Estimate based on regression of tarsus length and mass	-0.60	0.023
Age	Age in weeks since hatching	-0.28	0.096
Random Effects	**Variation**	**St. Deviation**	
Bird	2.53E-09	5.03E-05	
Captures	2.43E-02	1.56E-01	

Figure 2.1: A map of all rookeries from which nestling American white ibis (*Eudocimus albus*) were sampled in 2020 and 2021. Circles mark rookery locations, with green for natural rookeries and blue for urban rookeries.

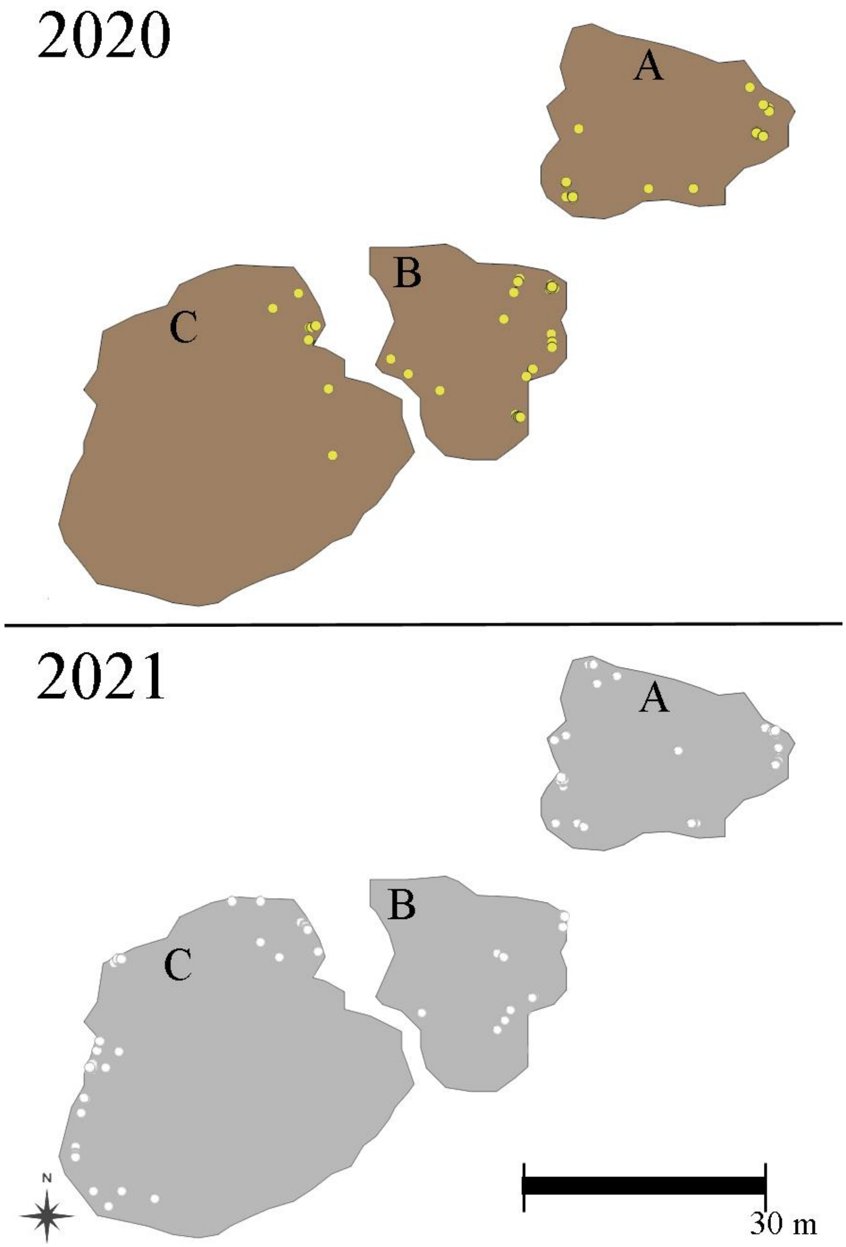

Figure 2.2: A map of an urban aquatic bird rookery in South Florida, composed of three tree islands, during two breeding seasons, 2020 and 2021. Islands, called A, B, and C, are located in a man-made body of water located between a golf course (southeast) and a residential area (northwest). Locations of marked American white ibis (*Eudocimus albus*) nests are indicated by circles.

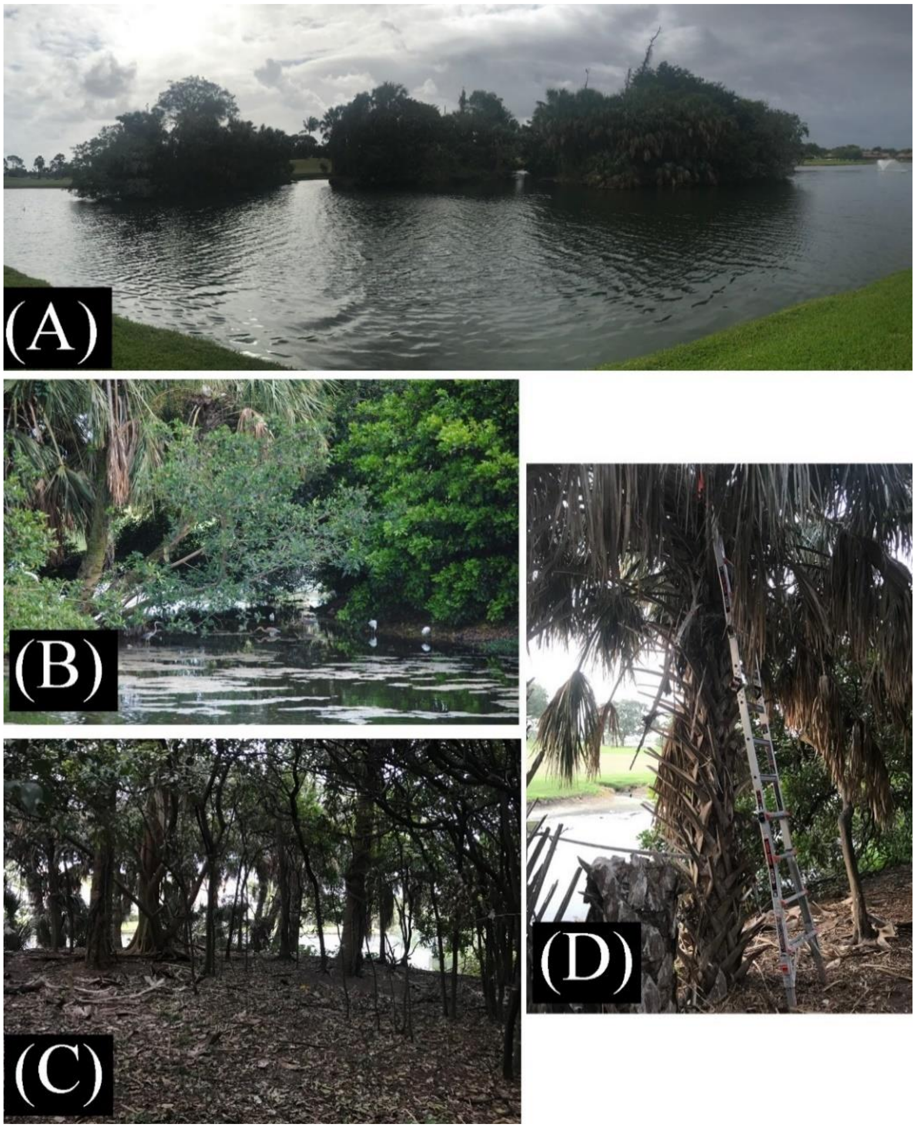

Figure 2.3: An urban aquatic bird rookery located on tree islands on a golf course community in Palm Beach County, FL, from which American white ibis (*Eudocimus albus*) nestlings were sampled repeatedly during the 2020 and 2021 breeding season. (A) Three islands, called A, B, and C from left to right, as viewed from the shore on the Northwestern residential side. (B) Close up of the crossing between B and C showing the boundary vegetation and water depth relative to adult ibis (white birds on right side). (C) The interior of rookery island A. (D) Ladder set up to access a marked ibis nest on rookery B.

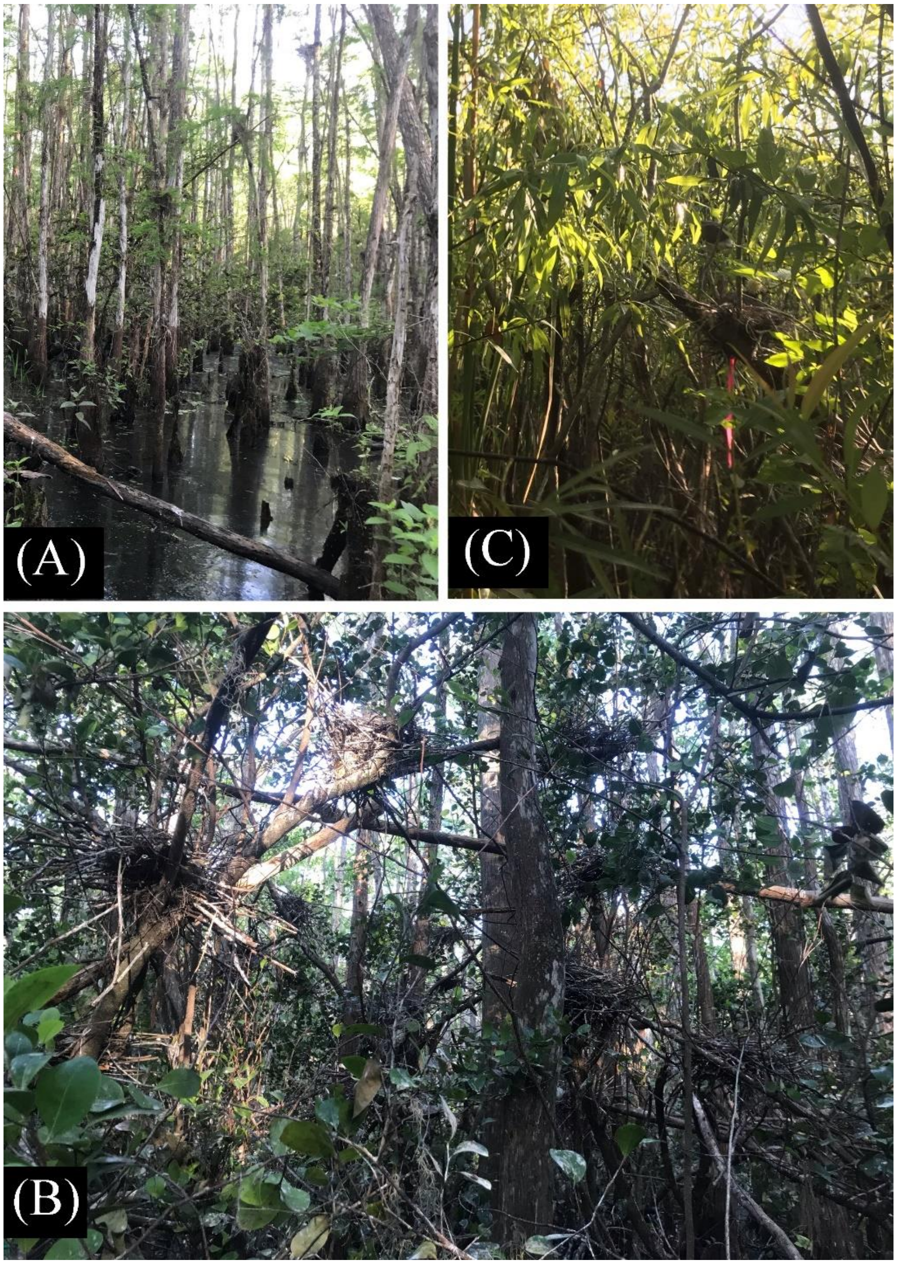

Figure 2.4: Two natural aquatic bird rookeries located in Water Conservation Area 3a in the Greater Everglades ecosystem from which American white ibis (*Eudocimus albus*) were sampled repeatedly during the 2021 breeding season. (A) Rookery site Hidden, showing the cypress swamp surroundings. (B) Rookery site Hidden, showing the interior including ibis nests. (C) Rookery site Alley North, showing the interior and a marked ibis nest.

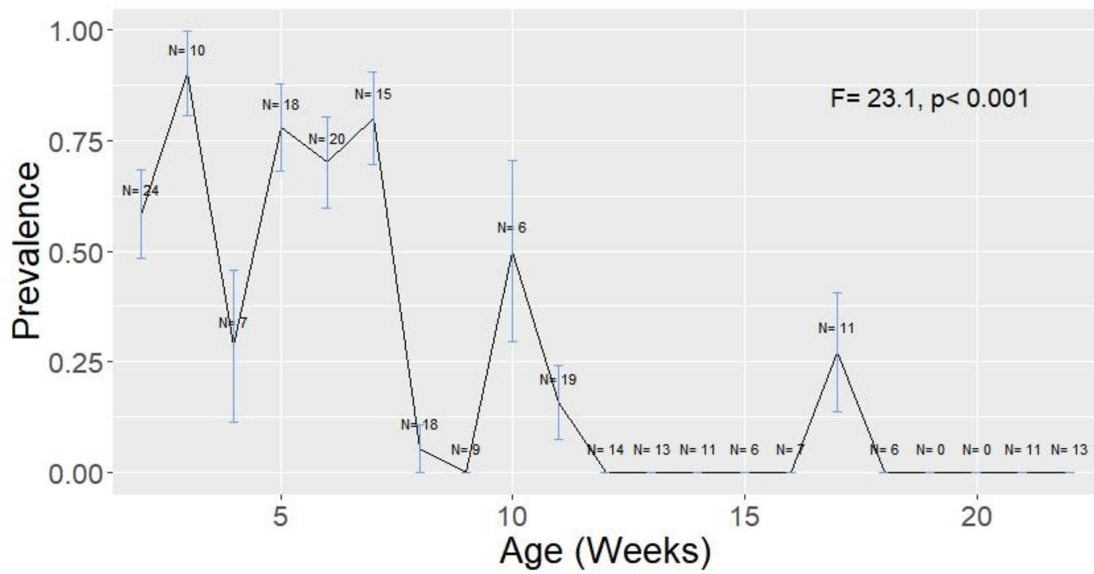

Figure 2.5: *Salmonella* prevalence of fecal samples from a captive colony of wild-caught American white ibis (*Eudocimus albus*) nestlings by weeks of age, from the time of capture at 2 weeks old until all birds consistently tested negative leading up to the experimental infection trial at 26 weeks. Error bars represent a standard error calculation based on the number of birds sampled that week, and *Salmonella* prevalence was confirmed by XLT-4 and ChromAgar analysis.

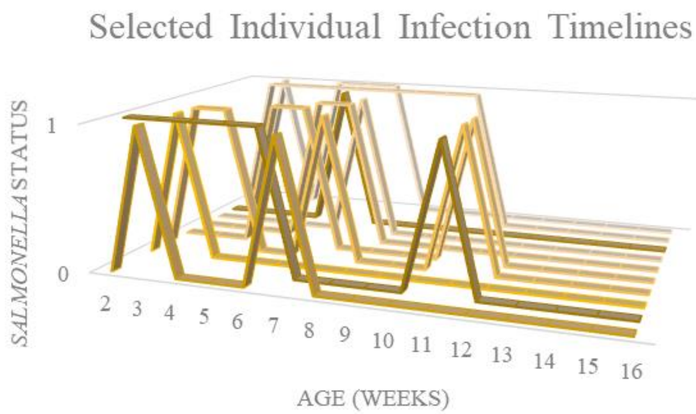

Figure 2.6: *Salmonella* status (shedding (1) or not shedding (0), as detected by XLT-4 and ChromAgar analyses of fecal samples) of a subset (N=10) of a captive colony (N=24) of American white ibis (*Eudocimus albus*) nestlings. Nestlings were wild-caught from South Florida at approximately 2 weeks old and monitored for 5 months. Fecal samples were collected at approximately one week intervals, revealing relatively high prevalence of nestlings during the first 7 weeks of life (67%, N=24), variable *Salmonella* shedding duration, and instances where birds stopped shedding and then began again.

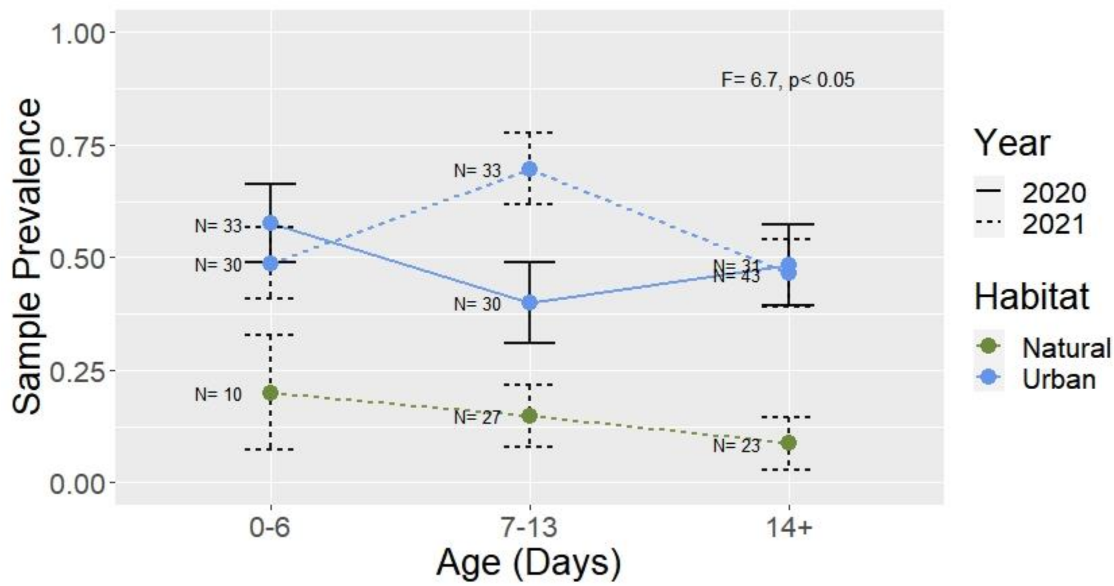

Figure 2.7: Prevalence of all fecal and fecal swab samples collected for *Salmonella* isolation from American white ibis (*Eudocimus albus*) nestlings in urban (2020 and 2021) and natural rookeries (2021), showing variation in the relationship between sample prevalence and nestling age across sites and years. Nestlings were sampled repeatedly up until approximately 3 weeks of age and *Salmonella* prevalence was confirmed through both XLT-4 and ChromAgar analyses. Error bars represent a standard error calculation and p-value is based on a model including the effects of year, habitat, and age group, though habitat is the only variable of this model that is independently significant.

CHAPTER 3

AVIAN INFLUENZA VIRUS PREVALENCE AND MATERNAL ANTIBODIES OF JUVENILE AND NESTLING AMERICAN WHITE IBIS (*EUDOCIMUS ALBUS*)

Introduction

Avian Influenza Virus in Wild Birds

Avian influenza viruses (AIV) are negative-sense single-stranded RNA viruses of the family Orthomyxoviridae, with viral subtypes identified by their combination of hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. Type A influenza viruses, unlike types B and C, are capable of infecting birds and some mammals, including humans, although susceptibility varies by species and viral strain. Subtypes HA1 through HA16 and NA1 through NA9 of type A influenza virus have been detected in migratory waterfowl and shorebirds (Webster et al. 1992). AIV is maintained in some avian populations, which likely serve as the ultimate genetic reservoir from which influenza in other taxa arises (Webster et al. 1992). The reassortment of surface proteins can yield subtypes which can evade existing humoral immunity. These reassortment events and other mutations occur by chance, and thus are more likely to occur when influenza prevalence is high and a large diversity of hosts and viral subtypes are present.

Large congregations of wild birds play an important role in the transmission cycle of AIV, as demonstrated by the documented reassortment events and poultry influenza outbreaks associated with migratory waterfowl (Humphreys et al. 2020; Tyson-Pello and Olsen 2020). Generally, detected influenza transmission in wildlife is increasing, possibly due to alterations in wildlife community assemblages and densities caused by human land use changes and climate change (Vandegrift et al. 2010; Wu et al. 2017), although increased awareness and surveillance may also be fueling this increase. These changes are often most evident around cities and agricultural areas, where novel selective pressures and landscapes contribute to species interactions and behaviors that would not have occurred in an undisturbed landscape (Shanahan et al. 2014). It is vital to understand the AIV dynamics of urban-adapted bird populations as well as the changes that occur as populations move from natural to urbanized zones, due to their higher likelihood of contact with humans and domestic animals in those spaces. While species in the orders *Anseriformes* and *Charadriiformes* are the most well-studied of the wild AIV reservoirs, other species may contribute to AIV maintenance and transmission in wildlife populations, though each species exhibits variation in susceptibility, shedding rates, seasonal prevalence patterns, and transmission likelihood (Webster et al. 1992; Stallknecht and Brown 2017; Tyson-Pello and Olsen 2020). Determining the unique components of the host-pathogen relationship for urban-adapted hosts can help us to predict the impact of urbanization on the pathogen dynamics of these species and the species with which they congregate.

The American White Ibis System

The American white ibis (*Eudocimus albus*) is a nomadic wading bird common across the Southeast. They are tactile foragers that use their long beaks to probe in shallow water (approximately 10 to 32 cm) or vegetation and locate their prey, often moving in loose flocks of varying size and species composition as they forage (Kushlan 1977a; Kushlan 1979, Binkley, Dorn, and Cook 2019). They commonly consume prey such as crayfish, insects, fish, and small amphibians, with diet composition dependent on their foraging habitat (Kushlan 1979; Kushlan, 1980). In the past 20 years, they have become common in urban parks, backyards and other spaces, foraging on terrestrial invertebrates and provisioned with anthropogenic food such as bread and other processed items. (Frederick et al. 1996; Hernandez et al., 2016).

Ibis tend to breed in large, mixed species rookeries during the spring and early summer, traditionally in large natural wetlands (Kushlan 1977b). In addition to populations of a number of other colony nesting aquatic bird species, these colonies can house as many as 60,000-100,000 ibis, which rely on large areas of land around the colony for foraging. As the need for prey is highest at this time, changes in the suitability of these foraging sites, such as changes in water level or prey availability, can cause shifts in breeding colony location and size (Kushlan 1977b; Frederick and Ogden 1997). Recently, ibis have begun breeding in smaller colonies found in urban areas, possibly driven by the consistent food source provided by human refuse and provisioning.

AIV in Ibis

Sympatric Reservoir Species

Characteristics of the ibis natural history (e.g. aquatic, breeding in dense colonies, nomadic, aggregation with other known host species) provide them with ecological opportunities to be natural reservoirs of AIV. Additionally, ibis share both urban and natural habitats with known AIV reservoirs in their breeding colonies and in urban parks and foraging areas. The most notable of these are waterfowl (*Anseriformes*), such as the mallard (*Anas platyrhynchos*) and mottled duck (*Anas fulvigula*), which ibis frequently mingle with in parks, and the black-bellied whistling duck (*Dendrocygna autumnalis*), which have been known to frequent the same urban rookery islands ibis are now breeding in. Mallards are considered the model species for AIV in wild birds and are the most common source of AIV isolates from wild ducks in North America (Hinshaw et al. 1985). AIV prevalence in this species typically reaches a peak, sometimes over 30%, in the late summer and fall, often attributed to the pre-migration congregation of large numbers of ducks, especially immunologically-vulnerable juveniles (Stallknecht 2003; Krauss et al. 2004; Latorre-Margalef et al. 2014). AIV has been isolated from all North American Anatidae duck species, including the black-bellied whistling duck, as well as the Egyptian goose (*Alopochen aegyptiaca*), which is an exotic waterfowl species found on urban rookery islands alongside the ibis (Stallknecht 2003). There is high subtype diversity in most duck species as well as high variability between populations, seasons, and years (Stallknecht 2003; Latorre-Margalef et al. 2014).

The other major avian group associated with AIV is shorebirds and gulls (*Charadriiformes*). The epidemiological pattern of AIV in shorebirds is different than in ducks, however, as shorebirds tend to experience two peaks in AIV prevalence, the larger of the two in the spring and one in the fall, associated with their migration periods, though the timeline varies somewhat by species (Kawaoka et al. 1988; Krauss et al. 2004; Maxted et al. 2012). Shorebirds also show high subtype diversity and some strains isolated from shorebirds have reduced pathogenicity in ducks, indicating some level of host adaptation (Kawaoka et al. 1988; Stallknecht 2003). Ibis cohabitate with gull and tern species, some of which breed in Florida and some of which winter there. Those common in the area for at least part of the year, such as the ring-billed gull (*Larus delawarensis*), often overlap with beach- and park-foraging coastal ibis. Other shorebird species, such as the red knot (*Calidris canutus rufa*) and the ruddy turnstone (*Arenaria interpres morinella*), overlap with ibis during their non-breeding season, along beaches and coastal marshes. AIV prevalence of red knots is typically relatively low (<2%; Maxted et al. 2012a), but their antibody prevalence is among the highest in wild shorebirds (90%, Johnson et al. 2014). Ruddy turnstones on the other hand tend to have high prevalence compared to other shorebirds during the spring peak (>10%; Maxted et al. 2012a; Maxted et al. 2012b).

AIV research on birds outside of these two groups is often less exhaustive, though some of the wading bird species ibis share habitat with, including the often-close quarters of a multispecies breeding colony, have been identified as potential AIV hosts or reservoirs (Stallknecht 2003). AIV has been isolated from the glossy ibis (*Plegadis falcinellus*) (Stallknecht Brown and Swayne 2008), cattle egret (*Bubulcus ibis*), black crowned night heron (*Nycticorax nycticorax*), and snowy egret (*Egretta thula*) (Siembieda et al. 2010), and some species have been found to be positive for AIV antibodies though no active infection has been detected, as is the case in the little egret (*Egretta garzetta*) and double crested cormorant (*Phalacrocorax auratu)* (Cross et al. 2013; Wang et al. 2014). AIV has been isolated in other heron and cormorant species in other parts of the world but is considered rare (Alexander 2000; Tracey et al. 2004). This indicates that, in some of these wading bird species that ibis might interact with, AIV infection is possible but, based on surveillance to date, at fairly low prevalence under natural conditions. Problems of unequal sampling effort and difficulty in AIV detection mean that the AIV status of some of the Florida wading bird community is unknown (Stallknecht, Brown, and Swayne 2007).

Ibis AIV Study to Date

Bahnson et al. showed that ibis are susceptible to experimental infection with AIV and are infected in natural populations (Bahnson et al. 2020). In their study, captive raised ibis juveniles were found to be susceptible to $10^{6.2}$ 50% egg infectious dose H6N1 and H11N9 AIV, inoculated via the choanal cleft. Virus was detected in water samples used by infected birds, and direct contact birds that had not been inoculated became infected, indicating the potential for both environmental and direct transmission among ibis. The average cloacal shedding period was 6 days for H6N1 and 5 days for H11N9, with neutralizing antibodies present in all inoculated and contact birds by 14 days post-inoculation. In the same study, Bahnson et al. failed to isolate AIV from any of the 118 oropharyngeal and cloacal swabs collected from free-living adult ibis. However, the observed antibody seroprevalence in these free-living ibis was about 70%, and, of these antibody-positive birds, over 80% had been exposed to multiple AIV HA subtypes. Combined, these results indicate that ibis are exposed to AIV in the wild, are susceptible to infection, and shed virus before seroconverting, but the exact timing and demographic information about when these infections take place is unknown. These missing pieces are crucial in understanding how AIV affects ibis at the population level and how this may change as a result of their increasing urban resource use.

Questions related to the role of ibis in AIV transmission hinge around the high antibody prevalence in free-living individuals. There is high variability in antibody production and titer among bird species infected with AIV; the exact antibody type and titer that is protective against subsequent infections remains unknown (Suarez and Schultz-Cherry 2000). Additionally, the persistence of antibodies is unknown; although the high AIV seroprevalence of ibis indicates that AIV infection took place, we do not know when in their life and annual cycles, or how often it has occurred. Study of the closely related Australian white ibis (*Threskiornis molucca*) has yielded similar results—relatively high seroprevalence to AIV in adults (average of 37% prevalence across multiple years) but no isolation of AIV in the wild (Epstein et al. 2006).

AIV is often difficult to detect when sampling wild bird populations due to the short temporal window for shedding, and many species exhibit distinct seasonal patterns that affect prevalence (Webster et al. 1992). To better understand when and to what extent American white ibis are affected by and may transmit AIV, we would need more frequent surveillance of individuals at all life stages to determine how and when ibis become infected. Two potential explanations are likely—either (1) ibis have frequent and continuous exposures to AIV with only short-term immunity, or (2) ibis experience a small number of predictable periods of exposure, perhaps as nestlings in dense breeding colonies or in their first years of winter influenza seasons, that lead to long-lasting immunity. This distinction in timeline, as well as any deviations that occur in urban populations versus natural ones, is important for evaluating pathogen risks posed by urban ibis populations and the effects of the pathogen on this rapidly-urbanizing species. If exposure is continual and not restricted to young birds in breeding colonies, viral shedding is also not restricted in time or location and could pose a risk to urban avian species and other birds that share habitat with the ibis throughout its range. If, on the other hand, exposure is through finite periods of high infection risk during the under-studied early life stages of the ibis, AIV could be a factor in their life history and population dynamics and any changes in AIV prevalence associated with urbanization would be of significance in understanding the effects of this shift in habitat use.

Significance of Nestling and Juvenile AIV Study

In this study, we sampled nestling and juvenile ibis, many of which were utilizing urban zones. These age groups have potential to be particularly vulnerable to infection due to their immune naivety, as demonstrated in juvenile mallards, which are a critical part of the AIV transmission cycle in their populations (Stallknecht and Brown 2017). Many of the AIV isolates from free-living wading birds have come from juveniles and nestlings in rookeries, indicating these may be hot spots for AIV detection (Siembieda et al. 2010).

Maternal Antibodies

This vulnerability may be offset in nestlings by the presence of maternal antibodies. Studies on the role of maternal antibodies on the transmission of AIV in wild birds are limited and primarily address antibody prevalence in egg yolks. In mallards and gulls, not all antibody-positive females will pass AIV antibodies to all eggs, though egg antibody prevalence is still relatively high and, in these species, correlates with local AIV virus and antibody prevalence (Pearce-Duvet et al. 2009; Hammouda et al. 2011; van Dijk, Mateman, & Klaassen 2014). The reasons for the within- and between- species variability in maternal antibody transfer are poorly understood, so the relationship between adult antibody prevalence and offspring maternal antibody acquisition needs to be understood in each species (Boulinier and Staszewski, 2008). In species where the correlation is close and reliable, egg sampling can be a convenient method of assessing adult antibody prevalence (Pearce-Duvet et al. 2009).

Maternal antibody persistence has commonly been assumed to be around 14 days in birds, but recent studies show that there is more variation than previously thought, and relatively long-lived, slow-maturing birds like ibis may have more persistent maternal antibodies and more investment in acquired immunity (Apanius 1998; Garnier et al. 2011). Though maternal antibodies are not protective against AIV infection in every species, they have been shown to impact pathogen transmission in multiple colony nesting birds including the kittiwake and albatross (Staszewski et al. 2007; Garnier et al. 2011). The protection conferred by these antibodies may also be adaptive for individuals, allowing hatchlings to allocate resources to other aspects of their health for those critical first weeks (Boulinier and Staszewski 2008). Thus, maternal antibody prevalence in ibis nestlings may contribute to reducing AIV transmission in breeding colonies.

Objectives and Hypotheses

Many factors impact ibis nestling health and infection status in their first weeks of life and must be fully understood in order to assess the contribution of immature age classes to the AIV transmission cycle. Age-specific factors, such as immune naivety, breeding colony conditions, and maternal antibodies add complexity to AIV epidemiology in these groups. As such, we aimed to determine the prevalence of AIV in nestling and juvenile ibis as well as investigate whether nestlings were conferred maternal antibodies to AIV. We sampled nestlings and juveniles in urban areas of Palm Beach County, Florida as well as nestlings from natural colonies in the Everglades Frances S. Taylor Wildlife Management Area. Analysis within and between these groups sheds light on the age-specific epidemiology of this pathogen as well as any changes that are occurring in urbanizing ibis populations versus those in natural areas. Fecal samples from nestlings and juveniles were collected to determine infection prevalence, and plasma samples were collected from nestlings to determine maternal antibody prevalence and persistence. We anticipated one of these two scenarios was most likely: either nestling prevalence would be non-zero and higher in the urban rookery than the natural one, indicating AIV infections are common during the nestling stage and are affected by the urban rookery conditions, or that nestlings may have high prevalence of maternal antibodies and thus not be commonly infected with AIV during their first weeks of life. In this case, we predicted that newly-independent young juvenile ibis in their first few winter influenza seasons may have high AIV infection prevalence as these protections fade and they are exposed to the wider landscape during foraging.

Methods

Study Sites

Urban Region and Rookery Site

We collected samples from nestling and juvenile American white ibis in two different areas, one urban and one natural. The locations of all sampling sites are shown in Figure 3.1. Urban nestlings and juveniles were sampled in Palm Beach County, Florida. Palm Beach County is within the Greater Everglades ecosystem and is home to urban populations of iconic Everglades fauna including alligators, raptors, and wading birds, and ibis are commonly sites in both developed and undeveloped areas of this county. It is the third most populated county in Florida, located on the southeastern coast. The population of the county has been steadily growing for the past century, with urban development in this area contributing to Florida’s loss of approximately half of its pre-statehood wetlands.

Nestling samples were collected from a rookery in a large golf community in Palm Beach Gardens, FL. Between April and July of 2020 (n=36) and 2021 (n= 42), we sampled birds from nests located on three adjacent islands within a body of water bordered by a residential community and a golf course (Figure 3.2). The islands, named A, B, and C, were approximately 542 m^2, 478 m^2, and 1312 m^2 in area, respectively, and composed of palm trees (approximately 10m tall), Ficus, Brazilian pepper, and other trees and shrubs. The islands had dense canopies with relatively open interiors (Figure 3.3A-D). Other aquatic bird species utilizing the islands included the little blue heron (*Egretta caerulea*), great egret (*Ardea alba*), and double-crested cormorant, as well as smaller groups of Egyptian geese, anhinga (*Anhinga anhinga*), glossy ibis, snowy egrets, black crowned night herons, tricolored herons (*Egretta tricolor*), and great blue herons (*Ardea herodias*). Potential predator species such as American crows (*Corvus brachyrhynchos*), blue jays (*Cyanocitta cristata*), and American alligators (*Alligator mississippiensis*) were observed in and around the islands.

Samples from juveniles were collected from Palm Beach County parks and neighborhoods frequented by ibis during December 2020 under the permits previously described. Locations included Dreher Park (n= 9), Kagan Park (n= 5), Indian Creek Park (n= 9), Bryant Park (n= 1), Lake Worth Beach Park (n= 1), and Chatham Hills neighborhood (n= 13), also shown in Figure 3.1. Other birds seen in the area included mallards, which often joined the group of feeding ibis. These locations were selected due to their status as common areas for people to feed birds, including ibis.

Natural Rookery Sites

Two natural-area rookeries (Figure 3.4A-C) in the Everglades Water Conservation Area 3a and Frances S. Taylor Wildlife Management Area in neighboring Broward and Miami-Dade counties were also sampled. Sampling took place from March to July of 2021 only. These rookeries, called Alley North and Hidden, have been well-studied and have been active for decades, though their populations and relative species compositions fluctuate. Alley North, approximately 50,000 m^2 in area and often the rookery with the largest population in this region, is comprised primarily of willow and cypress trees surrounded by cattails and sawgrass (Figure 3.4C). Many other wading bird species also use this island, including great egrets, roseate spoonbills (*Platalea ajaja*), glossy ibis, tricolored herons, and more. The site called Hidden, however, was dominated by white ibis during the 2021 breeding season, though some great egrets and small heron species were also present. Hidden is approximately 50,000 m^2 in area as well and the vegetation is dominated by cypress trees and cocoplums (Figure 3.4A-B).

Sampling Procedures

Urban Rookery

In the urban rookery, each of the three islands was visited on average once per week. Personnel entered the island by self-propelled boat at approximately 6:30 am and exited between two to five hours later. All nests that were within reach or that could be checked with a mirror pole were numbered and monitored weekly. Upon hatching and every week thereafter, the oldest nestlings from all accessible nests were captured by hand or using a wire-loop snare pole until the nestling was too mobile for capture, most commonly at three weeks of age. Sampled nestlings were banded and marked to ensure consistency across weeks, with plastic bands appropriate for bird leg size. These were changed weekly until a permanent band could be fitted. Age at time of sampling ranged from 1 to 25 days old.

Each week, choanal/cloacal swabs and blood samples were collected to track AIV virus and antibody prevalence over the first weeks of life. A sterile swab was used to collect a choanal/cloacal sample which was then stored in chilled viral transport media (VTM) containing 2mL Brain Heart Infusion (Becton Dickinson and Co. Sparks, MD) supplemented with penicillin G (1,000 units/mL), streptomycin (1 mg/mL), kanamycin (0.5 mg/mL), gentamicin (0.25 mg/mL), and amphotericin B (0.025 mg/mL) (Sigma Chemical Company, St. Louis, MO) antimicrobials. Blood was collected from the jugular or leg vein of each individual, not exceeding 1% of the body weight. Blood was centrifuged for 10 minutes at 3,300 rpm within six hours of collection and the plasma aliquoted before freezing. Samples were maintained at 4ºC in the field for no more than five hours and then either shipped overnight at 4ºC or frozen in liquid nitrogen until transport to the Southeastern Cooperative Wildlife Disease Study (SCWDS) in Athens, GA, where they were stored at -80ºC until processing.

The rookery access was granted under access permit PER 11-464, federal scientific collecting permit MB53675D-0, Florida scientific collecting permit LSSC-11-00119H, and UGIACUC Animal Use Permit A2019 10-009-Y1-A0

Natural Rookery

Similar procedures were followed at the natural rookery site and the above-mentioned permits applied to this location as well. Variations in sampling procedure from the urban rookery were as follows: these islands were accessed by airboat, the time on each island was restricted to one hour, no extendible ladders were used, and weekly checks began only after nestling hatching, not at egg laying. All samples were shipped overnight to SCWDS at 4ºC.

Urban Juvenile Sample Collection

Urban juvenile sampling took place over five non-consecutive days during December 2020 with an average of two hours of observation per location. Ibis flocks that included juveniles and were already habituated to feeding by people were provisioned with bread and freshly deposited fecal samples from juveniles collected opportunistically during the observation period. Juveniles were identified by their brown plumage, and approximate ages recorded based on the extent of the brown coloration. Samples collected represented birds in their first (n= 12), second (n= 10), and third (n= 16) year since hatching. Sterile swabs were used to collect samples and then stored in VTM. They were maintained at 4ºC for no more than five days and then shipped with frozen gel packs to SCWDS and stored at -80ºC until processing.

Avian Influenza Virus Analysis

AIV prevalence analysis was completed at SCWDS using virus isolation as previously described (Stallknecht et al. 1990). Briefly, choanal/cloacal swabs or fecal samples were thawed, vortexed, and centrifuged at 1500g for 15 minutes. Supernatant from each sample was inoculated (0.33 ml per egg) into three 9 – 11 day-old embryonated chicken eggs via the allantoic route. Eggs were incubated at 37ºC for 5 days; amnio-allantoic fluid was then harvested from each egg and tested in a hemagglutination assay using 0.5% chicken red blood cells (Killian 2008).

To test for the presence of virus molecules, viral RNA extraction was carried out on all swab samples using MagMAX-96 AI/ND Viral RNA Isolation kit (Ambion, Austin, TX, USA) on the Thermo Electron KingFisher magnetic particle processor (Thermo Electron Corporation, Waltham, MA, USA) (Das et al. 2009). All extracted RNA was analyzed using real-time reverse transcriptase PCR (rRT-PCR) targeting the matrix gene to confirm AIV presence or absence (Spackman et al. 2002).

Antibody detection

Nestling plasma samples were screened using a commercial blocking enzyme-linked immunosorbent assay (bELISA; FlockCheck AI MultiS-Screen Antibody Test Kit, IDEXX Laboratories, Westbrook, Maine, USA) as per the manufacturer's instructions. Briefly, each sample was tested in duplicate on separate plates, and absorbance at 650 nm was measured using an EMax Plus microplate reader (Molecular Devices, San Jose, CA, USA). Samples with sample-to-negative control (S/N) absorbance values $\geq$0.70 were considered negative for antibodies to AIV, and samples with S/N values $\leq$0.70 were considered positive (Brown et al. 2009, Shriner et al. 2016).

Data Analysis and Visualization

Data was processed using Microsoft Excel and visualized using Microsoft Excel and RStudio, with the RStudio 'ggplot2' package. Where needed, error was estimated using a standard error calculation.

Results

Urban and Natural Area Nestling AIV Prevalence and Serology

No viruses were isolated from any samples from the urban or natural ibis rookery in 2020 or 2021. One sample from a natural rookery nestling sampled at 6 days old in

2021 yielded a rRT-PCR-cycle threshold value of 33.98; viral RNA was not detected in any other swab or fecal samples. These 271 choanal/cloacal swab samples represented longitudinal sampling of 116 ibis nestlings (urban, 2020 N= 36; urban, 2021 N= 42, natural, 2021 N= 38) in their first weeks of life. Virus isolation and rRT-PCR results are shown in Table 3.1.

Based on bELISA results, 95% (N=101) of nestlings sampled were positive for AIV nucleoprotein antibodies at some point during sampling. Prevalence was high and fairly stable across sites and years, with prevalence at the urban rookery of 100% in 2020 (N=22) and 98% in 2021 (N=41), and prevalence at the natural rookery was 89% (N=38). Nearly all serially sampled birds displayed a pattern consistent with the presence and decay of maternal antibodies, with S/N ratios increasing with age (Figure 3.5). Overall sample prevalence was 83% (N= 229), as some birds became negative as they aged. Fewer birds were included in this analysis than in the virus isolation analysis because some plasma samples from 2020 were lost. A breakdown of the antibody prevalence, average number of captures and average sampling ages of the birds at each site and year is given in Table 3.2.

The persistence of these antibodies differed from nestling to nestling, with S/N ratios varying widely even within nestlings of the same age, as seen in Figure 3.5. For example, one nestling went from antibody-positive to antibody-negative by 8 days of age, while another nestling was still antibody-positive at 23 days of age. Given that many nestlings were still antibody-positive at their final sampling (70%, N= 101), the average age at which these birds catabolize their maternal antibodies cannot be determined, though it may to be greater than 2-3 weeks. The prevalence of antibodies among the birds sampled in this study, by age in days, is shown in Figure 3.6.

Juvenile AIV Prevalence

AIV was not isolated nor detected using rRT-PCR from 38 fecal samples collected from urban juvenile ibis (Table 3.1).

Discussion

Our findings indicate that ibis nestlings sampled throughout their first three weeks of life in urban and natural breeding colonies in Florida are not commonly infected with AIV. We also did not isolate virus from urban juvenile ibis during the winter. Ibis nestlings in both environments had high seroprevalence (95%, N=101) and persistence of maternal antibodies, which, paired with the absence of virus isolation, indicates that they may be protected from AIV infection. We cannot detect a habitat effect in this study because, although the prevalence of the natural rookery is lower than the prevalence of the urban rookery, the ages of birds sampled is different between the two habitats and thus cannot be directly compared, as age is a critical component of maternal antibody detection. These results also confirm the high levels of AIV infection in wild adult ibis, as nearly 100% of mother birds must have had circulating antibodies at the time of egg-laying to yield the prevalence of nestling antibodies detected. This is higher than the antibody prevalence previously measured in adult ibis (Bahnson et al. 2020), which may be due to a previously-undetected seasonal or demographic pattern in antibody prevalence. It is possible that the breeding season (variable, but typically from March to July) may be the period where adult infections are common, leading to the high antibody prevalence and high maternal antibody transference. This part of the year has rarely been included in adult sampling efforts due to the complications of mating and nesting, but infections in that time period may be the explanation for high nestling antibody prevalence. Alternatively, there may be a sex bias in adult antibody prevalence, with females having higher antibody levels that are then passed to offspring through the egg yolk, though this still leaves the question of when these adult infections occur unanswered. In either case, these findings reassert the consistent influenza exposure in adult ibis populations and bring us closer to understanding the annual and demographic trends that influence immunity and infection.

In general, the maternal AIV antibody prevalence estimates in this study are higher than in other wild aquatic birds, though studies of this type are rare. In the yellow-legged gull (*Larus michahellis*) and mallard, egg yolk antibody prevalence is lower than adult antibody prevalence at the same time and location, indicating not all antibody-positive females pass antibodies to their offspring (Hammouda et al., 2011; van Dijk, Mateman, & Klaassen 2014). In red knots, which have some of the highest known prevalence of AIV antibodies among shorebirds (>90%; Maxted et al. 2012a), sampled nestlings (<2 weeks old, N=16) have no antibodies, showing how variable maternal antibody passage is between species (Johnson et al. 2014). Female ibis in this study seem to pass antibodies readily and these antibodies can persist for multiple weeks. No nestlings were infected during these early weeks of life and their antibody prevalence and persistence was high. This could indicate ibis have a relatively high investment in humoral immunity to AIV and that the transfer of maternal antibodies to young is an important part of their interaction with the pathogen.

No virus was isolated from the juvenile ibis in this study. However, only 38 birds were sampled, in the winter. and it is possible that they were simply not shedding at the time, due to the experimentally demonstrated narrow window of AIV shedding in ibis (Bahnson et al., 2020). Ibis have a slow maturation rate and the juvenile period lasts approximately three years, so this age group necessitates further sampling to capture the short window during which an AIV infection could be detected. Additionally, 68% of those sampled were in their second or third winter, while first-winter birds would likely be the most immunologically vulnerable. Most of the juvenile birds we could access foraging in urban parks were between 1.5 and 2.5 years of age. If this pattern is consistent, new study sites may need to be located in order to find these first-year birds, although this age class is notoriously absent from easily accessible sites.

Our objective was to determine whether ibis are commonly infected with AIV during the nestling or juvenile stages of development, which could account for the high seroprevalence of AIV antibodies in adult ibis (Bahnson et al., 2020; Hernandez, unpublished data). We had two theories about how ibis may acquire such a high prevalence of antibodies: (1) ibis are infected frequently with AIV throughout life and repeatedly develop immunity to it or (2) they are infected at a specific life stage and maintain long term immunity after that point. Our virus isolation and serology results indicate that it is unlikely ibis commonly become infected as young nestlings. We also failed to isolate virus from urban juveniles, another potentially vulnerable age group, though our conclusion that this age group is not commonly infected is tentative. Thus, while this study does give us some direction for future work based on the maternal antibody findings, we have not yet determined the timing and frequency of AIV infection within the ibis life cycle or definitively eliminated the juvenile stage as a contender for the source of ibis AIV infections.

A similar gap in information exists for the Australian white ibis. Epstein et al. studied pathogens in this heavily urbanized species but was not able to isolate virus despite a 37% mean antibody prevalence in wild adults across multiple years of study (Epstein et al., 2006). In that study, juveniles had a significantly lower antibody prevalence (21%), which indicates that, in a species related to and sharing many ecological characteristics with the American white ibis, AIV infection and seroconversion may continue throughout the juvenile maturation period leading to this higher prevalence in adults. This shows the age specificity of the influenza cycle in these birds and indicates that the juvenile age group may be a vulnerable stage, though no active infections have been found in any sampling attempts from Australian white ibis.

This information on the timing of infections is of interest in American white ibis because it differs from other well-studied waterfowl AIV cycles and because of the nomadic nature of the ibis. Mallard ducks, the model species for AIV in wild birds, have a distinct infection timeline that includes high prevalence of infection during fall, especially among juveniles, with a predictable cycle from year to year (Stallknecht 2003; Krauss et al. 2004; Latorre-Margalef et al. 2014; Swayne 2016; Stallknecht and Brown 2017). A distinctive schedule of infection in ibis has yet to be determined, and we have yet to isolate an active AIV from an ibis. This is of concern as ibis have the opportunity to transmit to or be infected from multiple known AIV hosts, including mallards and other ducks and shorebirds with which they share both urban and natural habitat. Ibis forage and breed across the Southeast, including Louisiana, a state with large waterfowl aggregations, where they could potentially be influencing pathogen dynamics along the Mississippi flyway (Frederick et al. 1996). Recent concern over outbreaks of highly pathogen avian influenza (HPAI) in poultry have led to some increases in wild bird surveillance and the discovery that the natural AIV cycle may involve more wild birds than commonly thought, such as the Ciconiformes and Pelicaniformes species mentioned above (Stallknecht, Brown, and Swayne 2008; Deliberto et al. 2009). As such, understanding the AIV dynamics of ibis remains important to understanding how AIV cycles in wild birds in critical habitats and how this may change as urbanization alters the way host species live and form communities.

To better understand AIV infection dynamics in ibis populations, future work should target the age group between nestlings and older juveniles, especially in the light of the juvenile involvement in the AIV dynamics of other waterfowl. Additionally, adults at breeding sites during the spring and summer should be sampled to determine if infections are common in this time period as may be indicated by the high levels of maternal antibodies found in ibis nestlings. As such, though the frequency and life history stage of ibis AIV infection is still unknown, the information gleaned from this detailed study helps fill epidemiological gaps and provide guidance for continued work in understanding this host-pathogen relationship.

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Tables and Figures

Table 3.1: Choanal/cloacal swab samples collected for avian influena virus (AIV) isolation and rRT-PCR analysis from American white ibis (*Eudocimus albus*) nestlings in two rookeries in the 2020 and 2021 breeding seasons and from ibis juveniles in urban parks. Nestlings were sampled repeatedly up until approximately 3 weeks of age, and juveniles were sampled only once. Virus isolation was done by egg passage and hemagglutination assay, and rRT-PCR was targeted to AIV matrix protein.

Year	Habitat Type	Age	N Birds *(N Samples)*	AIV Virus Isolation	rRT-PCR *(Ct value)*
2020	Urban	Nestling	36 *(94)*	0	0 *(n/a)*
	Urban	Juvenile	38 *(38)*	0	0 *(n/a)*
2021	Urban	Nestling	42 *(114)*	0	0 *(n/a)*
	Natural	Nestling	38 *(61)*	0	1 *(33.98)*

Table 3.2: Avian influenza virus (AIV) nucleoprotein antibody prevalence based on bELISA of American white ibis (*Eudocimus albus*) nestlings sampled iteratively from rookeries in urban and natural areas in South Florida during the 2020 and 2021 breeding seasons. The mean number of captures and age range at which birds were sampled at each site and year is also displayed.

Year	Habitat Type	Antibody Prevalence	Mean Number of Captures	Mean Age (Days) at First Capture *(Range)*	Mean Age (Days) at Last Capture *(Range)*
2020	Urban	1.00	2.14	3.8 *(1-21)*	13.18 *(1-24)*
2021	Urban	0.98	2.74	3.14 *(1-9)*	16.17 *(1-25)*
	Natural	0.89	1.74	9.61 *(1-23)*	14.55 *(1-23)*

Figures

Figure 3.1: A map of all locations where nestling and juvenile American white ibis (*Eudocimus albus*) were sampled in 2020 and 2021. Large circles mark rookery locations, with green for natural rookeries and blue for urban rookeries, and small red stars mark locations where juvenile ibis were sampled.

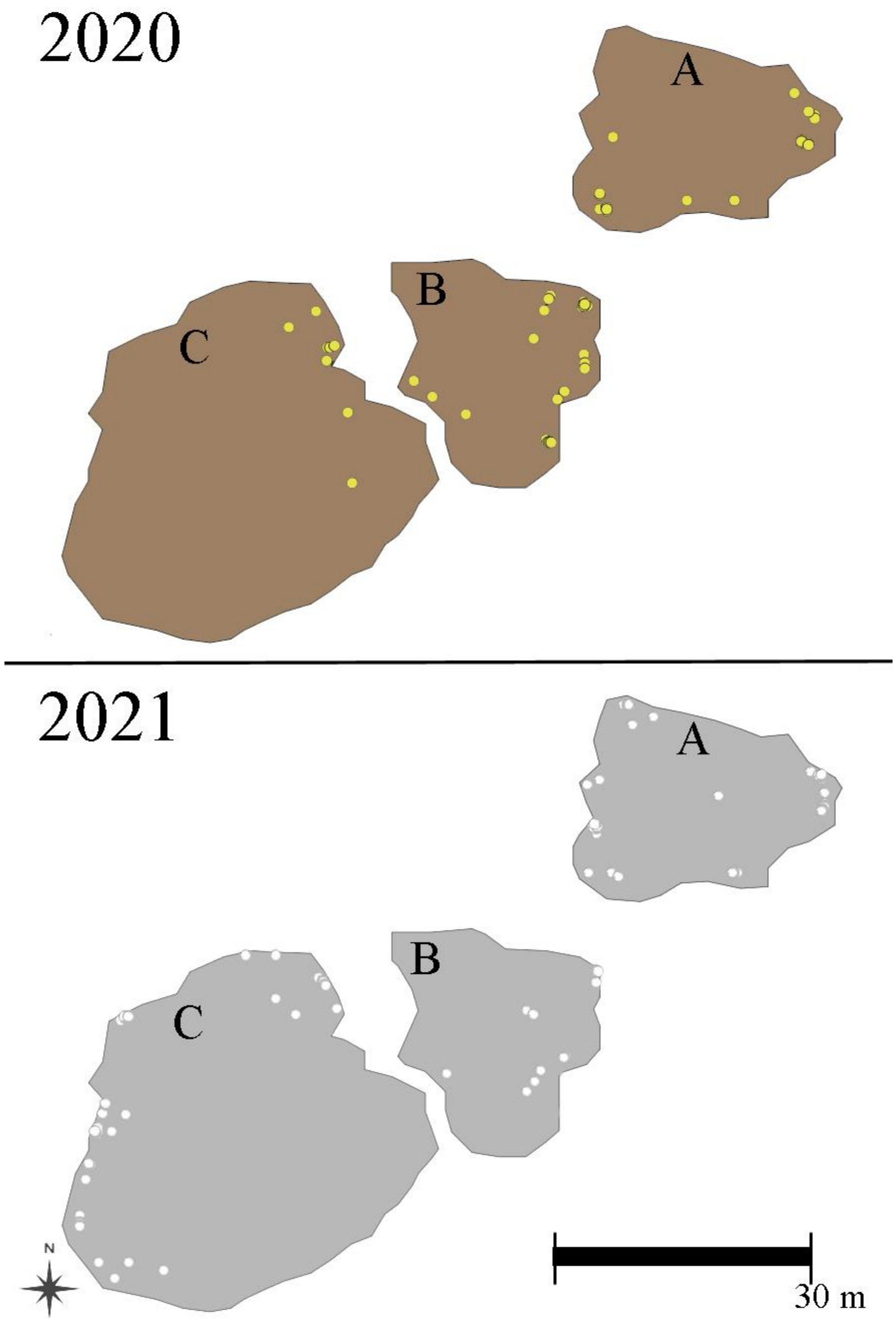

Figure 3.2: A map of an urban aquatic bird rookery in South Florida, composed of three tree islands, during two breeding seasons, 2020 and 2021. Islands, called A, B, and C, are located in a man-made body of water located between a golf course (southeast) and a residential area (northwest). Locations of marked American white ibis (*Eudocimus albus*) nests are indicated by circles.

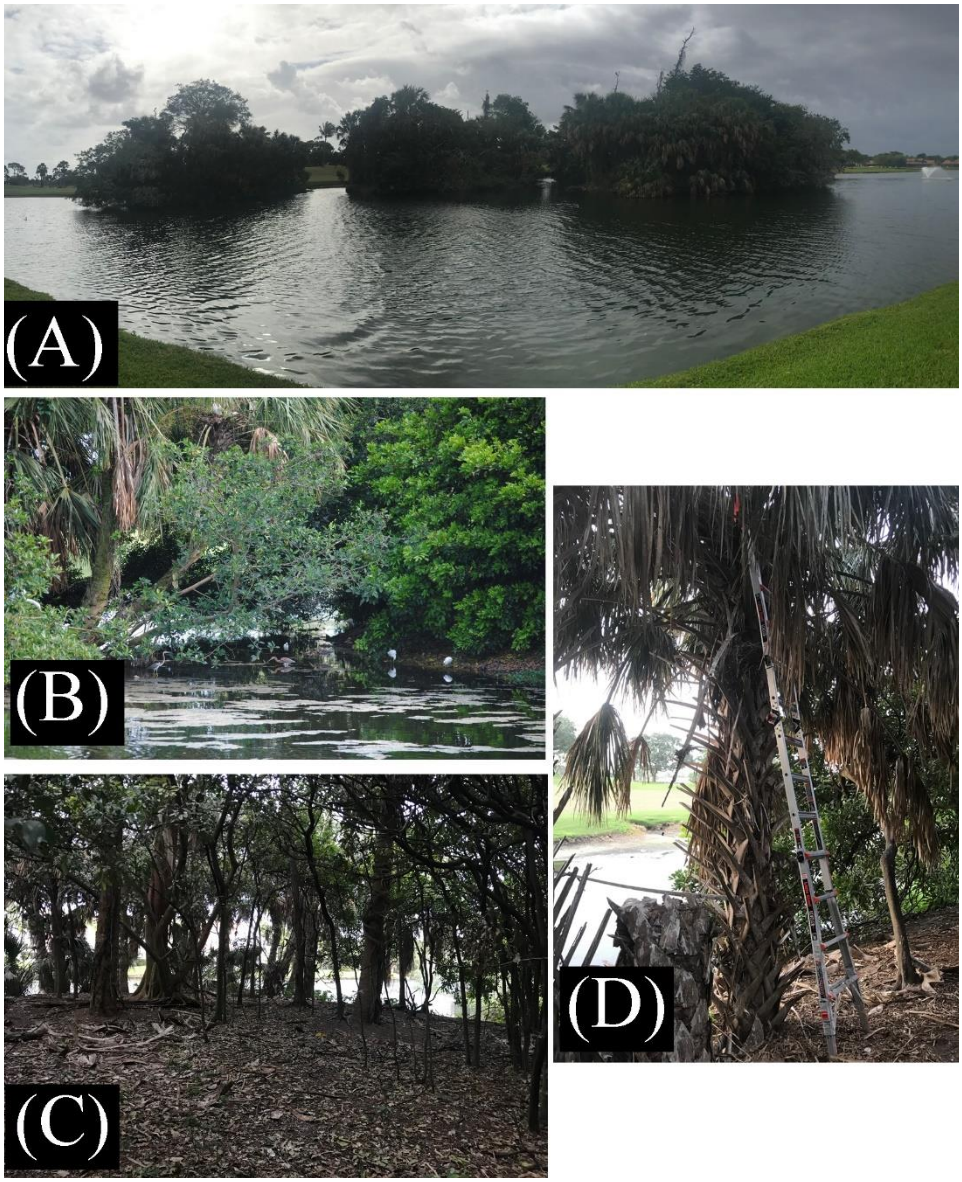

Figure 3.3: An urban aquatic bird rookery located on tree islands on a golf course community in Palm Beach County, FL. (A) The three islands, A, B, and C from left to right, as viewed from the shore on the Northwestern residential side. (B) A close up of the crossing between B and C showing the boundary vegetation and water depth relative to adult American white ibis (*Eudocimus albus*). (C) The interior of rookery island A. (D) A ladder set up to access a marked ibis nest on rookery B.

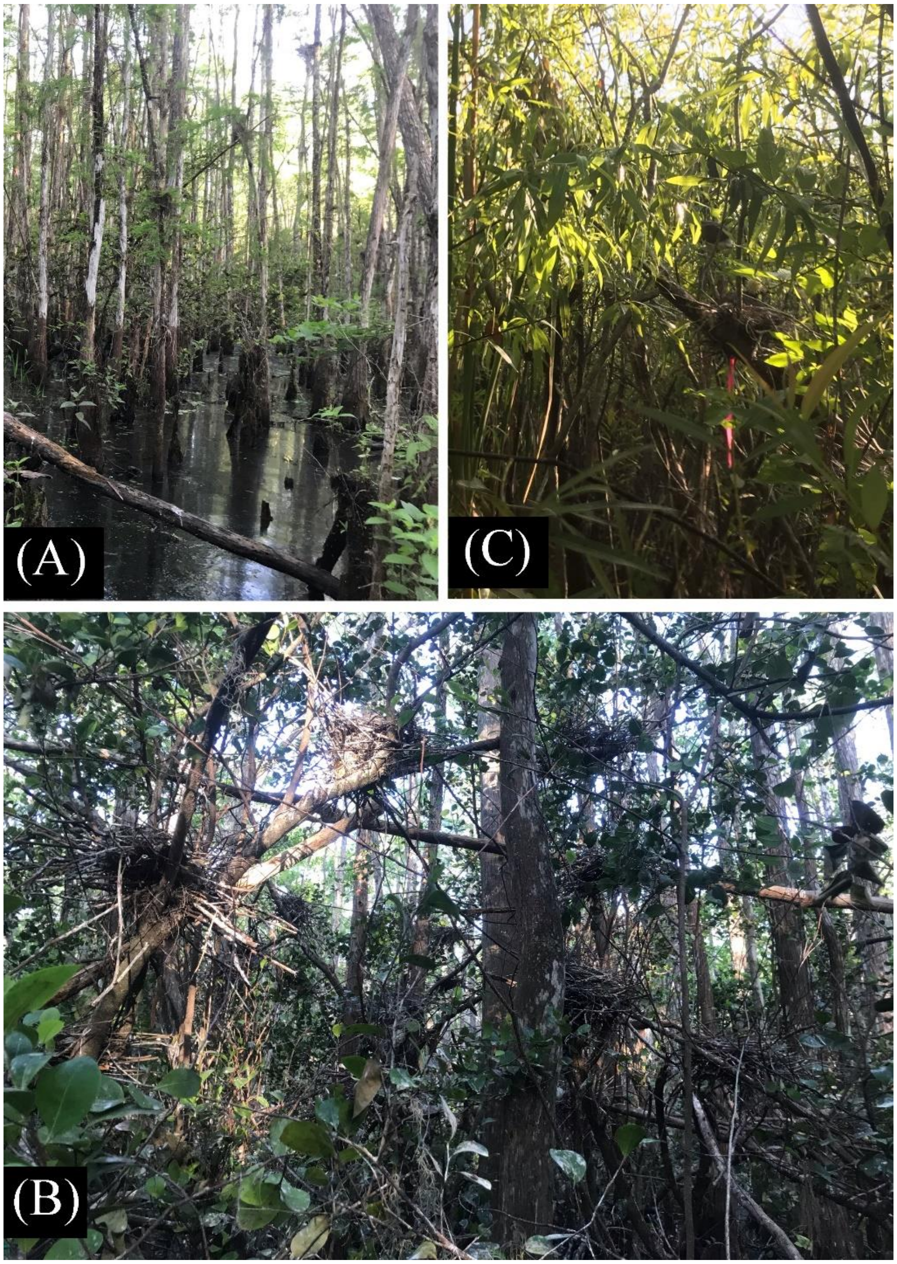

Figure 3.4: Two natural aquatic bird rookeries located in Water Conservation Area 3a in the Greater Everglades ecosystem. (A) Rookery site Hidden, showing the cypress swamp surroundings. (B) Rookery site Hidden, showing the interior including American white ibis (*Eudocimus albus*) nests. (C) Rookery site Alley North, showing the interior and a marked ibis nest.

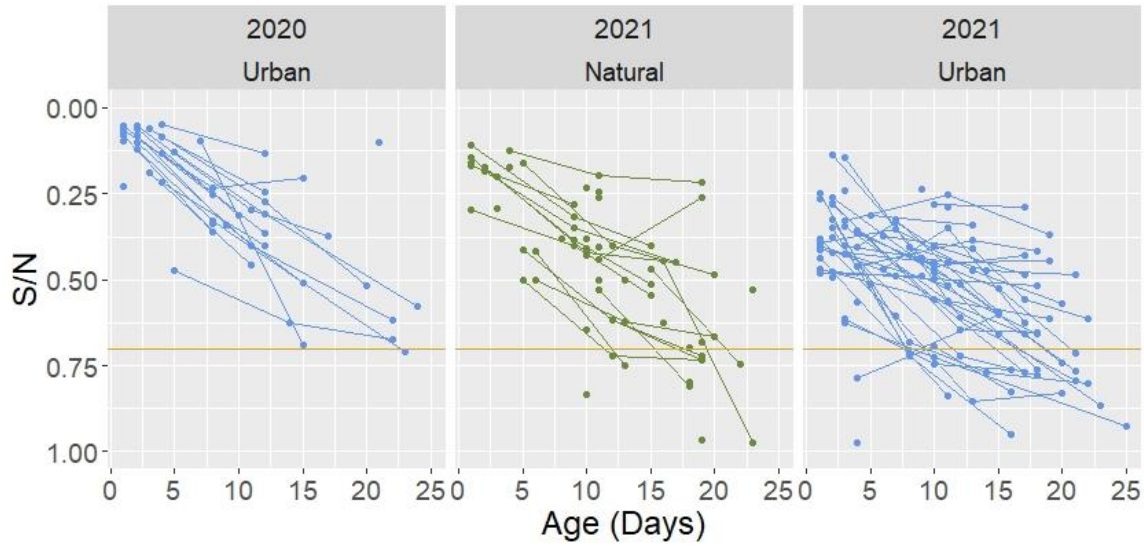

Figure 3.5: Sample to negative (S/N) ratio (displayed on a reversed Y axis for easier interpretation) for plasma samples (N= 229) of American white ibis (*Eudocimus albus*) nestlings across the 2020 and 2021 breeding seasons in a South Florida urban rookery and from the 2021 breeding season in natural rookeries, also in South Florida. The yellow line denotes the threshold for negativity, with S/N values below that line considered negative. Samples taken from the same bird at multiple ages are connected by a line, while samples taken from birds that were not serially sampled are only a dot.

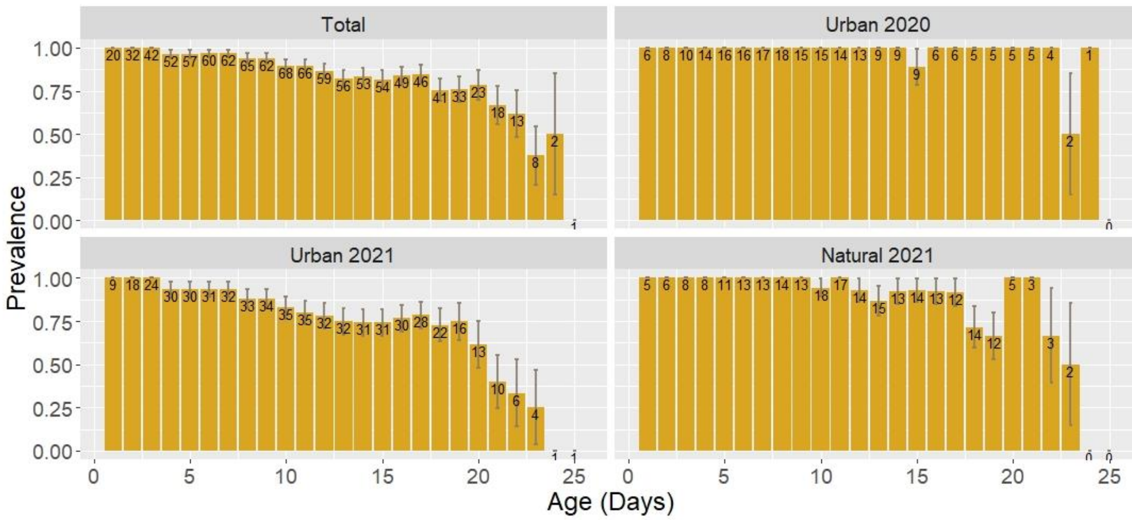

Figure 3.6: The prevalence (proportion of individuals sampled with sample to negative (S/N) values below 0.7) of avian influenza antibodies in American white ibis (*Eudocimus albus*) nestlings sampled in the 2020 and 2021 breeding seasons, from urban and natural rookeries in South Florida. The numbers within the bars represent the number of sampled birds through that age (in days). Birds that tested positive were assumed to remain positive until the occurrence of a bELISA S/N ratio above the 0.7 threshold. Error bars represent a standard error calculation.

CHAPTER 4

CONCLUSION

Summary of Results

The American white ibis (*Eudocimus albus*) is experiencing rapid ecological changes as urbanization affects the landscapes where it was once so numerous. The movement into urban zones creates opportunities for altered avian disease dynamics, which may combine with other ecological shifts to change the way this species functions in the ecosystem. Our exploration of the age-specific epidemiology of avian influenza virus (AIV) and *Salmonella* in the ibis fills in yet another component of the complex ecology of this iconic wading bird species. The lack of AIV isolation from nestlings or juveniles paired with the high prevalence and persistence of maternal antibodies of nestlings indicates that younger ibis are not likely involved in the cycle of AIV transmission in their populations and communities. Additionally, this study of maternal antibody decay over time across multiple sites and years may provide us with new means of understanding this process in wild aquatic birds, many of which have only been studied using egg yolk antibody detection.

Contrastingly, our study confirms that nestling ibis are particularly vulnerable to infection with *Salmonella*, a fact which had been established in a previous study (Hernandez et al. 2016), and expands our knowledge of what that vulnerability means for these birds. Nestlings from both urban and natural sites as well as those raised in captivity had high prevalence of *Salmonella* beginning at a young age and some birds shed virus for multiple weeks. None, save one bird in the experimental infection trial in the captive colony, displayed signs of clinical disease, and *Salmonella* was not connected to any mortality events in the urban colony in 2020, despite 81% of sampled birds shedding *Salmonella*. The urban colony had significantly higher *Salmonella* prevalence than the natural colony, indicating that the urban landscape around the colonies affects pathogen transmission, either directly or indirectly through their parents. Whether or not urban areas facilitate a similar elevation of AIV transmission is still unclear.

Limitations

We had initially planned to sample nestlings in both the urban and natural colonies for two breeding seasons, but limitations of access due to the COVID-19 pandemic prevented us from entering the natural area rookeries in 2020. As such, our sample size of natural area nestlings is lower and we only have one year of comparison, however this one year does include birds from geographically separate rookeries and our information from the urban rookery indicates little year to year variation in the pathogens studied. The lowered capture likelihood in the natural rookery, however, provides further limitation to this data set, as birds were often captured only one or two times with an average age at first capture of 10 days old, compared to 3 days old in the natural rookery. This was in part due time constraints for visiting these natural colonies, which prevented us from locating and monitoring nests prior to nestling hatching, and due to more difficult mobility on these densely vegetated islands. Our statistical model of *Salmonella* controls for this difference, but this disparity in ages sampled made urban versus natural analysis of the AIV antibody results impossible, since prevalence is so strongly tied to the ability to sample birds in their first week of life. The differences in travel time and access to these different rookery sites may also have led to small variations in sample storage, though consistency was maintained as much as field conditions would allow.

Future Directions

The study of these two pathogens yielded very different conclusions about nestling involvement in pathogen transmission. In AIV, as nestlings from the time of hatching until three weeks of age had 0% prevalence and maternal antibody retention, this group can be effectively ruled out as a common component of AIV transmission in ibis. Further sampling of juveniles, preferably including serology analysis, which would also explore whether infection is common among older nestlings whose maternal antibodies have been catabolized, is required before fully ruling out that age class. The high antibody prevalence of adults may not be due to age-class-defined infections, however, and may be due to intermittent, short lived infections in mature ibis. This epidemiological pattern may prove difficult to detect and will require expansive sampling efforts but using the information from this study of maternal antibody transfer and focusing on pre-breeding season adults first could yield more information about when and how AIV infections occur in ibis.

Our study of *Salmonella* also generated additional questions related to the duration of Salmonella resistance after infection, the process of urban pathogen transmission, and the effects of such high Salmonella prevalence on ibis nestlings and the species around them, including humans. Previous work on the ibis has shown that adults have an approximately 26% prevalence of *Salmonella* (Hernandez et al. 2016), a fact which now seems incongruous with the much-reduced infectivity and shedding period of experimentally infected birds who had recovered from a previous *Salmonella* infection.

This apparent protection identified in the trial may fade over time or be differentially effective across individuals, and understanding the mechanism by which this occurs would illuminate the ways that *Salmonella* immunity and reinfection play out in wild birds. Additionally, it would be beneficial to understand the specific cause of the elevated *Salmonella* prevalence in urban nestlings as it may have other effects on nestling health. The relative roles of exposure through parents, contact with infected nestlings, contact with other species, and altered health due to diet or stress play in facilitating this high prevalence could give us an indication of the long-term effects of these changes as urban breeding becomes more common. Finally, the identification of any sublethal effects of *Salmonella* infection of nestlings as well as whether these urban colonies increase the risk of spillover transmission to humans or other urban wildlife will be critical in understanding the full implications of this change in pathogen dynamics among urban-bred nestlings.

References

Hernandez, Sonia M., Catharine N. Welch, Valerie E. Peters, Erin K. Lipp, Shannon Curry, Michael J. Yabsley, Susan Sanchez et al. "Urbanized white ibises (Eudocimus albus) as carriers of Salmonella enterica of significance to public health and wildlife." *PLoS One* 11, no. 10 (2016): e0164402.