THE EFFECTS OF URBANIZATION AND AN ANTHROPOGENIC DIET ON STRESS AND IMMUNE FUNCTION IN AMERICAN WHITE IBIS (*EUDOCIMUS ALBUS*)

by

CAROLINE RUTH CUMMINGS

(Under the Directions of Kristen J. Navara)

ABSTRACT

We investigated the effects of urbanization and resource provisioning on immunity and stress in the American white ibis. We sampled birds captured in the field in Palm Beach County, FL, USA and assessed an individual's physiology based on the habitat they were captured in and the amount of provisioned resources they consumed as determined by stable isotope analysis in a concurrent study. We also raised ibis nestlings in captivity and tested how a simulated anthropogenic diet affects immunity and baseline stress levels. Birds captured from urban habitats had higher bactericidal capacity against E. coli, and adults from urban habitats had an attenuated stress response. Ibises consuming more provisioned resources had better bactericidal capacity against E. coli, lower baseline corticosterone levels in the non-breeding season, and a stronger stress response in the breeding season. A simulated anthropogenic diet had no effect on stress or immunity in white ibis nestlings.

INDEX WORDS: urbanization, urban wildlife, white ibis, *Eudocimus albus*, anthropogenic food provisioning, stress, immunity, corticosterone, bacterial killing, bird feeding, heat shock proteins, nestlings, diet manipulation

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Urban Habitats and Associated Changes

The world's population is expected to increase by four billion people over the next 85 years (Agarwal et al. 2018). Parallel to this growth in population, urbanization and expansion of populations around city centers has also increased, as the majority of people in developed countries continue to inhabit urban areas. Urban sprawl has resulted in the conversion of natural wildlife habitats to human-dominated land types. The land needed to support cities is 565 -1130 times higher than the physical area covered by the city itself (for various uses such as resource consumption and waste production), with this land coming from forest, agricultural, marine, and wetland ecosystems (Faulkner 2004). For instance, from 1950 to 1990, Chicago's population grew by 38% but spread over 124% more land (Donnelly et al. 2001). This encroachment affects the natural ecology of the landscape causing a shift in biodiversity and biomass, habitat fragmentation and loss, resource depletion, and an increased exposure of humans to wildlife (Faulkner 2004). Because North America is considered the world's most (sub)urbanized region, research on the effects, both positive and negative, of this shift in land-use on wildlife is imperative for creating management and conservation plans for species, especially those of concern (*2010 Census Urban and Rural Classification and Urban Area Criteria* 2010).

Urban habitats offer a range of opportunities and/or risks for wildlife and have been colonized by various, now adapted, species (e.g. rock doves, squirrels, etc). Additionally, wildlife not traditionally associated with urban environments may now increasingly use urban habitats (Beissinger & Osborne 1982) at least sporadically, due to available resources that urban environments provide. The success of a given species in a particular urban habitat is variable, but most urban environments offer some potential benefits to wildlife such as shelter, breeding habitats, access to supplemental food and water, and even reduced competition and/or predation for some species (McKinney 2006; French et al. 2008). However, urban habitats do not guarantee success for wildlife. The abundance and consistency of anthropogenic resources can lead to aggregations of urban wildlife in numbers that may contribute to changes in behavior (e.g. increased inter- and intra-species aggression and competition), changes to physiology driven by diet quality and quantity, and introduction of novel pathogens through contact and resource contamination (Murray, Becker, et al. 2016; Ryan & Partan 2014; Bradley & Altizer 2007).

Urban habitats are associated with a suite of stressors to which wildlife must acclimate in order to successfully exploit urban environments. As previously mentioned, altered community and population dynamics may contribute to changes in wildlife stress responses due to shifted competition and/or hierarchical dynamics, the potential for aggressive encounters with novel species or humans, and changes in predation risks as well as pathogen dynamics (i.e. urban cats preying on birds) (Lepczyk et al. 2004). Additionally, urban environments feature a collection of anthropogenic stressors such as increased ambient light and noise levels (Slabbekoorn & Ripmeester 2008); increased presence of and interactions with humans, including actions that may be considered harmful or agitating (e.g. chasing) (L. Nelson 2008); potential for harm caused by vehicles both on the land and in the water (DeWoody et al.); and exposure to novel contaminants and/or pathogens (Bradley & Altizer 2007). Experiencing these stressors can contribute to shifts in urban wildlife behaviors, both to their benefit and detriment. Constant interactions with humans may lead to wildlife becoming aggressive towards them, as seen in urban black kites in Japan (Galbreath et al. 2014). On the other hand, urbanization may lead wildlife to acclimate to human presence. For example, urban house sparrows had smaller flight initiation distances and a faster habitation to human disturbance than rural house sparrows (Vincze et al. 2016) and adult Magellanic penguins reduced the number of defensive head turns as human visitation increased (Brian G Walker et al. 2006). Conversely, wildlife may reduce or limit their activity in human-dominated areas and/or at human-dominated times or avoid them altogether, which has been well-documented in urban bobcats, coyotes, mule deer, and mountain lions (George & Crooks 2006; Tigas et al. 2002; Riley et al. 2003). In addition to habituation to or avoidance of human disturbance, certain characteristics of urban habitats can affect other aspects of wildlife behaviors. For example, an increase in artificial lighting at night affects the reproductive, migratory, foraging, sleep, and territorial behaviors of several mammals and birds (Stone et al. 2009; Kempenaers et al. 2010; Le Tallec et al. 2013). Foraging behavior may also be altered by urban resources and/or dependence on provisioned foods. As examples, Florida scrub-jays foraging in suburban areas were found to be more efficient than their rural counterparts due to access to provisioned foods (Fleischer et al. 2003) and aggregated resources contributed to reduced home range sizes in urban raccoons and baboons (Prange et al. 2004; Hoffman & O'Riain 2011).

These behavioral responses and habituation to novel urban stressors vary greatly by species, and can determine whether a species is capable of existing and succeeding in urban habitats. Furthermore, these behavioral shifts may complement or signify underlying physiological differences in urban wildlife, as behavior is often a reflection of physiology. In the case of wildlife adapting poorly to urbanization, individuals may respond so frequently to acute stressors in urban habitats that they enter a state of chronic stress and down-regulate their subsequent acute stress responses (Rich & Romero 2005). When this occurs, the hyperactive stress response may lead to the allocation of energy away from other physiological processes (John C. Wingfield et al. 1998). Existing in a state of chronic stress and an energetic deficit can have detrimental impacts on an individual's condition, reproduction, and immune function, all of which influence wildlife health (Padgett & Glaser 2003; Kleist et al. 2018).

An Introduction to Resource Provisioning

Despite the aforementioned changes in stressors and overall population and community dynamics, some species still exist and even thrive in urban habitats. A primary reason is because urban areas often provide key resources for survival, including supplemental food resources, both direct and indirect, such as backyard bird feeders, dumpsters and household garbage cans, and direct anthropogenic handouts such as park visitors feeding waterfowl bread (Malpass et al. 2017; Murray, Hill, et al. 2016; Welsh et al. 2017; Kristan III et al. 2004). All physiological processes require energy, and more consistent resources may promote fitness in urban habitats, particularly when the energy needed for physiological processes intensifies (e.g. during reproduction, or when mounting an immune response) (R. J. Nelson 2004). For instance, the cost of mounting an immune response in blue tits was low when birds had access to *ad libitum* food, potentially compensating for any energetic cost of the immune response through food intake (Peter et al. 2011; Amat et al. 2007; Nilsson 2002). Additionally, because food availability is the most important factor in determining an animal's activity budget, the presence of anthropogenic resources may decrease the necessity for extensive foraging, thereby saving time and energy for some species (Orams 2002; Lowry et al. 2013). The increase in energy uptake and decrease in energetic requirements may lead to the allocation of more energy towards processes such as immune function, self-care, or reproduction, and improve overall condition (Nilsson 2002).

The quality of resources in urban environments varies greatly from natural habitats and even across a gradient of urbanization. Some foods are considered appropriate supplementation for urban wildlife and have no negative reported consequences on health. For example, black-capped chickadees feeding on supplemented birdseed had improved survival (Brittingham & Temple 1988), common raven nestlings with access to anthropogenic resources fledged more successfully (Webb et al. 2004) and banded mongooses with access to supplemental resources carried more fetuses and had higher body condition (Otali & Gilchrist 2004). However, despite the caloric benefit, the quality of anthropogenic resources can be inappropriate and nutritionally insufficient to be considered beneficial. Feeding of fish using inappropriate food may result in fatty deposits on the liver, potentially leading to mortality (Orams 2002); urban coyotes consuming lower quality food (as determined by protein content) were more likely to be infested with mange (St. Clair et al. 2015); suburban crow nestlings consuming anthropogenic foods were found to be nutrient-restricted and their growth rates were lower than in rural nestlings (Heiss et al. 2009). Provisioned food may also be contaminated or toxic and therefore may introduce novel pathogens to the consumer, all having potential deleterious effects on wildlife health, including immune function (Wilcoxen et al. 2015). Wild bird seed, which has been shown to carry aflatoxins, illustrates this relationship, as it was shown to cause immunosuppression, cellular damage, and mortality in northern bobwhite chicks (Henke et al. 2001; D. Moore et al. 2013). Like many of the other effects of urbanization, though, the effects of supplemental feeding are species-specific. For instance, Bókony et al. (2012) found no negative effects of urbanization on body condition in house sparrows, despite receiving provisioned resources, but Murray, Kidd, et al. (in press) found a negative relationship between increasingly urbanized habitat/urban resource exploitation and body condition in American white ibis.

Urban Wildlife Health & Physiology

According to Stephen (2014), healthy organisms are capable of maintaining physiological processes at a rate which promotes their long-term fitness, despite exposure to immune challenges, such as pathogens or contaminants, and stressors, both acute and chronic. Critical to an individual maintaining its health is recognizing and resolving any "abnormal" situations; this process is typically mediated by the individual's immune system, which recognizes and defends against the suite of immune challenges it might face. The immune system is associated with a host of other physiological processes related to maintaining health, including the endocrine system, which responds to stressors to promote survival. Additionally, all physiological processes are dependent on energy, some even requiring specific nutrients; therefore, if an organism enters into an unhealthy state—either by infection or otherwise—energy is typically allocated away from processes deemed temporarily unnecessary and towards those most vital to their health (e.g. immune function in the case of infection) (I. T. Moore & Hopkins 2009). In the case of individuals who are not consuming enough energy to maintain healthy physiology, immune function may be down-regulated, contributing to increased infection and disease risks (Moret & Schmid-Hempel 2000). This may also be the case for individuals experiencing an energy-intensive event such as reproduction or frequent and/or intense stressors. The factors that influence an organism's health such as their diet, encounters with stressors, life history stage, and pathogen exposure can all be affected by their environment, particularly when shifting from natural to urbanized habitats because of the numerous changes associated with this shift. The immune and endocrine systems are such vital components to wildlife health, so it is important to understand how they function in relation to each other and are potentially affected by changes associated with urban habitats.

The Stress Response

When an organism encounters and responds to a stressor—defined as "any physical or psychological event that disrupts homeostasis"—it undergoes a sequence of physiological processes including the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Sheriff et al. 2011). The HPA axis mainly functions to maintain energetic balance in the face of shifting metabolic demands, and in the case of stress, acts to restore homeostasis through the release of glucocorticoids (e.g. corticosterone in birds), steroid hormones that help an animal appropriately respond to a stressor. The HPA axis is composed of the hypothalamic paraventricular nucleus (PVN), the anterior pituitary gland, and the adrenal cortex. Upon encountering a stressor, an animal's PVN is stimulated and initiates a cascade of physiological events that results in a measurable increase in circulating glucocorticoids within 3 to 5 minutes (in vertebrates). Within 15 to 30 minutes after initiation, glucocorticoids typically reach peak levels then begin to inhibit any further HPA response through a negative feedback loop, allowing glucocorticoid levels to return to baseline within 60 to 90 minutes (Sheriff et al. 2011). Once circulating, glucocorticoids bind to receptors throughout the body and act to up- or down-regulate a host of physiological processes relevant to responding to a stressor and promote survival, referred to as the "emergency life history stage" (e.g. energy mobilization and re-allocation via gluconeogenesis, activation or suppression of certain behaviors, immune regulation) (Wilcoxen et al. 2011; John C. Wingfield et al. 1998). The HPA axis and glucocorticoid release work in conjunction with other endocrine processes such as release of catecholamines (e.g. epinephrine and norepinephrine) to optimize an individual's response to a stressor. This response is considered normal, and with adequate energy intake, animals can allocate energy towards this response without experiencing adverse effects. Though varied by species and sometimes even individual, the amplitude and duration of this stress response often correlates with the overall health of an animal (Sheriff et al. 2011).

Maintaining this normal response to stress is referred to by McEwen and Wingfield as *allostasis* (McEwen & Wingfield 2003). Allostasis emphasizes an individual's ability to achieve stability in response to changes, both predictable and unpredictable. It differs from homeostasis in that it refers to an animal maintaining the balance of systems in differing ways depending on predictable factors such as current life history stage (e.g. breeding, molting, migrating) and less predictable factors such as environmental conditions (e.g. unfavorable weather patterns) and social context (e.g. unexpected aggressive interaction). The allostatic load, or current and predicted energetic demands facing an organism, dictates an individual's ability to maintain allostasis and therefore remain healthy. While homeostasis does not necessarily anticipate change, allostasis does, and wildlife are able to maintain allostasis as long as they have access to abundant energy resources or their emergency life history stage allows for a regain of positive energy balance through aforementioned changes. If energetic demands exceed what can be mobilized from stores (aka. Type I allostatic overload), and an individual's emergency life history stage does not reduce allostatic load, health can be detrimentally affected (e.g. diving petrels had decreased body mass and higher stress hormone levels in stormy weather) (G. T. Smith et al. 1994). Type I allostatic overload can occur when an individual encounters frequent and/or long-lasting stressors and subsequently enter a state of chronic stress. Continuous activation of the HPA-axis leads to the dampening and eventual cessation of the negative feedback loop which results in high circulating levels of glucocorticoids; this can lead to a cascade of negative effects on health. Energy is available is then diverted to the constant energetic demands of an activated HPA axis, becoming unavailable for use elsewhere (i.e. other physiological processes). In addition to the consequences of continually diverting energy to the chronic activation of the HPA axis, glucocorticoid concentrations can have deleterious effects on fitness due to their involvement with a variety of physiological processes, such as suppression of reproduction, digestion, and immune function, effectively reducing fitness and survival (Boonstra et al. 1998; J. Blas et al. 2007; Acevedo-Whitehouse & Duffus 2009). However, not every individual that encounters constant stressors reaches a state of chronic stress (Boonstra & Fox 2013). The ability to suppress the HPA response prior to reaching a chronic-stress state can prevent the suppression of immune function and other processes affected by the resulting upregulated levels of glucocorticoids.

In the context of shifting diets and habitats, the stress response and possibility of an animal reaching a state of chronic stress is imperative to their health and survival. Due to the accompanying environmental and social changes associated with urban habitats, allostatic load is nearly guaranteed to change within them and may parallel changes (either positive or negative) in stress physiology. Bonier (2012) concludes that a bird's exposure to and perception of stressors in an urban environment may affect their HPA axis and subsequently their glucocorticoid levels, which act as the primary mediators of the allostatic state. Urbanization and accompanying changes in allostatic load may affect the acute stress response of an individual via several factors such as resource availability or habituation to perturbations (e.g. interactions with humans). For example, if an urban environment increases allostatic load via frequent encounters with acute stressors, we might expect to see parallels in the stress physiology of that individual; birds may respond more acutely to stressors, increasing their glucocorticoid levels after an acute stressor, such as capture and handling (Matson et al. 2006). If frequent or intense enough, stressors may initiate a state of chronic stress, as previously mentioned, and baseline glucocorticoid levels may be high but response to an acute stressor may be low; for example, towards the end of a nutritional stress experiment, black-legged kittiwake chicks fed low-lipid diets had higher baseline stress hormone levels and a suppression of HPA activity in response to acute stressors (Kitaysky et al. 1999). This is especially relevant in urban environments, when resources may not be of high enough quality to promote positive energy balance, like the low-lipid diets the kittiwake chicks were consuming. Contrastingly, certain individuals may habituate to acute stressors and therefore show a dampened HPA-response in response to acute stressors. For example, adult hoatzins exposed to tourist disturbance responded less frequently to experimental stressors than adults at undisturbed sites (Müllner et al. 2004). Additionally, abundant resources in urban habitats may promote the efficient return to a "normal" state after activation of the emergency life history stage. Though not definitive or necessarily causal, investigation into an animal's stress response may contribute to classifying a species as an urban avoider, adapter, or exploiter and help us determine and predict the effects of urbanization on wildlife health.

Common Metrics of Stress

The most commonly used field indices to measure an individual's stress response are plasma glucocorticoid (GC) levels, which can be measured for both establishing a baseline and after inducing stress to measure the magnitude of the stress response. The effects of urbanization on GC levels (both baseline and stress-induced) vary dramatically across species and even individuals (see Bonier 2012 for a review of the most current urban GC literature in birds). While measuring GCs is useful, it is not allencompassing, and results can be difficult to interpret. Unfortunately, there is no definite consensus on what a high baseline GC levels or responses mean in terms of fitness, and several hypotheses present in the literature make interpretations difficult (see Bonier et al. (2009)). For example, though Romero and Wikelski (2002) determined baseline levels of Galapagos marine iguanas visited by tourists to be an indicator they were not chronically stressed, whether their reduced acute stress response was decidedly beneficial or harmful has yet to be determined.

Another common metric of stress is the quantity of heat shock proteins (HSPs) present in a sample. HSPs act as molecular chaperones, aid in protein folding, and help maintain protein stability, and are therefore present in normal cells. However, in the presence of a variety of stressors, HSPs are produced in greater quantities to protect cells from damage. Stressors that HSPs may respond to include temperature, infectious diseases, toxins, parasites, inbreeding, habitat conditions, and nutrition (Sørensen 2010; Herring & Gawlik 2007). Conversely to GC levels, HSP changes occur less rapidly, making them a useful indicator of long-term or chronic stress. Additionally, unlike GC levels, HSPs may be independent of capture stress. To illustrate the role of HSPs, Herring et al. (2011) found certain HSPs increased in American white ibis nestlings experiencing a decline in food availability, indicating an induction of stress via resource restriction. Moreno et al. (2002) found that smaller pied flycatcher nestlings had increased HSP levels, as well as increased heterophil/lymphocyte (H/L) ratios, indicating greater levels of stress. Martinez-Padilla et al. (2004) found HSPs to be a useful indicator of competitive stress in Eurasian kestrel nestlings, with nestlings that showed the largest size difference with respect to their largest sibling also showing higher levels of HSPs.

Immunity

Nearly all organisms are exposed to a wide range of pathogens and parasites in their environments; to defend against these pathogens, animals have evolved complex and multifaceted physiological responses to the presence of pathogens, including the two arms of the immune system: innate and adaptive immunity. In general, innate immunity is ever-present, reacts rapidly, and is non-specific—it acts as the first line of defense against invasion. There are several components that make up innate immunity: physical barriers such as skin and mucous membranes, resident flora such as non-pathogenic gut bacteria, humoral factors such as the complement system, and cellular responses like phagocytosis, inflammatory mediation, and the activation of natural killer cells. Adaptive immunity, which is typically slower in response, consists of a pathogen-specific response to invasion after an initial activation through the production and release of antibodies. Generally, the goal of the immune system is to differentiate between "self" and "non-self" substances and eliminate "non-self" invaders, especially those capable of damaging the host. Which arm or even specific component of an arm of the immune system that is used to launch a defense against an invader varies depending on the specific invader. Additionally, the specific response that is launched will depend on the host's energetic state, circulating stress hormones, and environmental conditions. Individual variation may also influence the immune response. Immunocompetence, or the optimal functioning of all the components of the immune system, is vital for wildlife health (Lochmiller 1996). Demonstrated by a wide range of studies, immune-deficient or –suppressed individuals exhibit decreased wound healing, higher mortality rates, shorter lifespans, and develop more severe infections and cancers (Gilbertson et al. 2003; Acevedo-Whitehouse & Duffus 2009).

The field of ecological immunology— "ecoimmunology"—recognizes that both extrinsic factors (e.g. environmental conditions) and intrinsic factors (e.g. energetic state) lead to changes in immunity and can therefore affect host health. Thus, ecoimmunological studies often assess the energetic tradeoffs associated with host immune function and the processes that affect these tradeoffs, such as entering a reproductive state, a change in diet quality, or increased stressful encounters. When considering the transition from natural habitats to more urbanized ones, these tradeoffs can play a vital role in the success of an individual or species in urban habitats and should therefore be adequately understood. As mentioned in the previous section, a suite of changes occurs when transitioning to urban habitats and these all have the potential to affect host immune function separately or in conjunction with each other. Changes associated with weather, diet, and stressors have been studied to determine their effects on immune function, sometimes through the mediation of the stress response. Chronic activation of the stress response can lead to immunosuppression, however, acute activation of the HPA axis has been linked to enhanced immune function to react to an unpredictable event (Martin 2009); for example, rainbow trout exposed to acute handling stress had enhanced lysozyme activity, an indication of a well-functioning innate immune system (Demers & Bayne 1997). However, tradeoffs may not apply equally to all components of immune function and are context specific (Norris & Evans 2000; Viney et al. 2005). Bourgeon and Raclot (2007) found that during a period of fasting (a nutritional stressor), the innate immune response of female common eiders was maintained, while cellular and humoral adaptive components were negatively affected. Bourgeon and Raclot (2006) and Stier et al. (2009) found that administration of corticosterone did not affect T cell-mediated immunity of common eiders or constitutive innate immunity of wild barn owl nestlings, but it did suppress humoral immunity in both species.

In addition to the above changes, pathogen dynamics also change in urban environments, which can impact a host's immune function (Bradley & Altizer 2007; Stier et al. 2009). Factors like disturbance or fragmentation of habitats and climate change can potentially lead to the emergence of pathogens in novel environments as well as affect pathogenesis (Gottdenker et al. 2014; Bradley & Altizer 2007; Martin et al. 2010). Additionally, human-driven or non-native transport of novel pathogens into a habitat can lead to changes in pathogen dynamics within the community, particularly for native wildlife (Dobson & Foufopoulos 2001). Lastly, resource provisioning in urban habitats can affect pathogen dynamics in several ways, both positively and negatively. Supplemental resources may increase condition and provide enough energy to improve defense against pathogens; conversely, the quality of provisioned food may be too low and therefore suppress immune function, making the host more susceptible to infection (see Murray, Becker, et al. (2016) for complete review). Supplemental feeding can lead to abnormal aggregations of wildlife, increasing contact and potentially the transmission of pathogens within a community (Becker, Streicker, & Altizer 2015). In addition to high densities, a more sedentary lifestyle brought on by resource provisioning can increase the likelihood of infectious stages accumulating and therefore promote pathogen transmission (Murray, Becker, et al. 2016).

Common Metrics of Immunity

The metric(s) chosen to elucidate immune function in wildlife depends greatly on the species, the component of the immune system being investigated, and the invasiveness of the procedure or assay. Many wildlife studies are purely observational because manipulation of wildlife health is difficult to do in the field. For these reasons, assays that rely on blood collection and storage for later analysis in a laboratory setting are most commonly used; examples of these include assessing bactericidal capacity (which are a proxy for innate immunity through complement proteins) via bacterial killing assays, cell counts including H/L ratio, phagocytosis assays (innate immunity by macrophages), and serum and plasma protein levels and the ratio of specific fractions, such as immunoglobulins (Norris & Evans 2000). To date, few studies have assessed bactericidal capacity in a strictly "urban versus natural" context, however, studies have shown considerable variability by species, age, sex, condition, life stage, and environmental conditions, as well as after experimental administration of corticosterone. For example, Matson et al. (2006) (five species of tropical birds) and Gao et al. (2017) (house sparrows) found corticosterone depresses immune function, Schmidt et al. (2015) (song sparrows) found sex- and bacteria-specific responses, Fratto et al. (2014) (house finches) found co-infection buffered the depression of bactericidal capacity with elevated corticosterone levels, and Merrill et al. (2014) (red-winged blackbirds) found a positive relationship between corticosterone and bactericidal capacity. On their own, bactericidal capacity, cell counts, or assessment of phagocytosis provide little insight into immune function due to their great variability and inability to assess the immune system as a whole. However, in conjunction with studies of other physiological parameters (such as stress), these assays may contribute to our understanding of urban wildlife. More invasive assays include the phytohemagglutin (PHA) challenge, lipopolysaccharide (LPS) challenge, or sheep red blood cell (SRBC) challenge. All of these include the introduction of a novel, non-toxic substance into the body and subsequent assessment of the immune response. PHA challenges assess the inflammatory response (T-cell dependent), LPS challenges assess the febrile response, and SRBC challenges assess an individual's ability to produce antibodies against an antigen (adaptive). Iglesias-Carrasco et al. (2017) found that out of four amphibian species tested, there were no differences in immune response to PHA in individuals from urban and natural populations, diets supplemented with carotenoids enhanced immunity in response to PHA and SRBC challenges in male zebra finches (Blount et al. 2003; McGraw & Ardia 2003), and high-protein diets improved immunity against PHA in northern bobwhites, but did not affect immunity against an SRBC challenge (Lochmiller et al. 1993).

Why Stress and Immune Function?

As discussed, urban habitats have the potential to both positively and negatively affect wildlife health. Habitat, food resources, exposure to, perception of, and response to novel stressors, exposure to novel contaminants and/or pathogens, and changes in behavior leading to changes in energetic input and expenditure are all interconnected and contribute to the success of an individual in urban habitats. Many of these factors are also correlated to physiological measurements of an individual's health such as physical measurements of condition, stress response (e.g. GC levels), immune function (e.g. bactericidal capacity, PHA, LPS, etc.), or pathogen prevalence. Therefore, integrative studies assessing these various aspects of wildlife health in urban environments can greatly contribute to understanding how a species is affected by urbanization and whether it is suited for success in urban habitats or capable of adapting to them in the future.

American White Ibises (*Eudocimus albus*)

American white ibis are wading birds of the family Threskiornithidae and order Pelecaniformes commonly found in wetlands along the coast of the southeastern United States and into parts of Central America and the Caribbean. Semi-nomadic and tactile foragers, white ibis move between wetland habitats in search of optimal water levels and prey (Bancroft et al. 2002; Kushlan 1986). With their long, down-curved bill, ibis tactilely forage by sweeping their heads and probing at the ground in search of prey; because of this foraging strategy, ibis require a specific depth of water and soft soil to successfully forage (Kushlan 1977). Additionally, prey abundance has been shown to be directly influenced by water levels (Kushlan 1979). Natural prey include many aquatic invertebrates, fish, and both aquatic and terrestrial insects (Kushlan 1979).

White ibis have historically been found in high numbers in Florida, USA, and are typically seen as synonymous with the Everglades; however, in recent decades, their nesting pairs in FL have declined dramatically (Frederick et al. 2009). Frederick et al. (1996) reported a 90% decline in breeding ibis since the 1930s and white ibis are now designated as a Species of Concern in FL (Crozier & Gawlik 2003). This is likely in response to the degradation of in Florida, particularly the Everglades ecosystem,—up to a 50% loss in size has been estimated, mostly due to agriculture and urban development (Chimney & Goforth 2001). Many of the driving factors of this phenomenon are anthropogenic—development, contamination, and draining of wetlands have led to hydrologic changes and habitat destruction of the ecosystem (Dorn et al. 2011). As a result of the changes to the wetland ecosystem, natural prey abundance for the American white ibis is less abundant, and birds can now commonly be found foraging elsewhere (Boyle et al. 2014; Dorn et al. 2011). Populations of white ibis are increasingly found foraging in urban habitats such as parks, residential lawns, landfills, and zoos. Here, the bulk of their food consumption is directly due to anthropogenic sources such as bread handouts in parks or anthropogenic food waste from dumpsters/landfills (Boyle et al. 2014; Murray et al. 2018). They may also maintain their natural foraging behavior and consume terrestrial invertebrates and aquatic organisms living in urban water sources; however, some flocks appear to be dependent solely on human handouts and supplemental resources (Welch 2016). The quantity and quality of supplemental food varies across urban landscapes, in addition to the species and stressors ibis encounter (Murray et al. 2018). In the most urbanized areas, ibis may exhibit behavioral shifts such as increased habituation to humans to the point where they eat out of people's hands (personal observation), extreme boldness (stealing food from people) or even aggression. They also encounter novel species such as established invasive species (e.g. muscovy ducks (*Cairina moschata)*), peridomestic ducks (e.g. American pekin ducks (*Anas platyrhynchos*)), and native species with which they would have little direct contact in natural environments at high densities (e.g. laughing gulls (*Leucophaeus atricilla*), boat-tailed grackles (*Quiscalus major*) and common gallinules (*Gallinula galeata*) (Welch 2016). Additionally, supplemental provisioning may be a driving factor for ibis site fidelity to urban habitats (Murray, Hernandez, et al. in press).

Previous Studies

Foraging in urban habitats not only leads to changes in diet composition but exposes ibis to the aforementioned urban-associated changes, such as shifts in stressors and pathogen dynamics. The effects of these shifts are not well-understood, and previous or ongoing studies have shown varying effects of urbanization and provisioned diets on American white ibis. Curry (2017) assessed multiple parameters of stress and immune function in urban ibis found some ibis to exhibit stress and/or immune levels indicative of either acclimatization to urban habitats or chronic stress, however, the results showed significant variation among urban birds. Murray et al. (2018) found a negative relationship between urbanization and body condition, likely a result of shifts in dietary quality, as birds captured from more urbanized sites consumed a diet higher in urban resources; Hernandez et al. (2016) reported a higher prevalence of *Salmonella* spp. in urban ibis. These results indicate ibis health may respond negatively to urbanization, which has implications for the species conservation and management as well as conservation of their natural habitats. However, Murray et al. (2018) found lower ectoparasite scores in ibis captured in more urban habitats, indicating potential tradeoffs in ibis physiology when foraging in urban areas. In fact, reliable access to supplemental resources (or other urban-associated benefits) may allow urban ibis to commit more time to preening (i.e. to remove ectoparasites). To fully understand the effects of urbanization and anthropogenic resources on American white ibis health and whether urban areas can act as suitable alternative habitats for ibis, it is imperative to integrate studies of health parameters and ibis behavior and movement; as we have seen, urbanization presents many tradeoffs for wildlife, and single measures of health and/or fitness are inadequate to predict a species' success in urban habitats.

Significance

Because American white ibis have recently transitioned into urban habitats in such high numbers, yet breed in natural habitats, their situation provides us with a unique opportunity to study urban wildlife and the mechanism(s) by which they either fail in, adapt to, or exploit urban habitats. Urban environments may provide opportunity for wildlife, yet there are also potential consequences that cannot be ignored;

assessing how ibis stress physiology and immune function varies by habitat can help elucidate the effects of urbanization as a whole and contribute to conservation strategies.

Objectives, & Hypotheses

Curry (2017) conducted a study assessing the stress physiology and immune function in white ibis captured in urban sites throughout Palm Beach County, FL. This study showed that though bactericidal capacity was not correlated with any stress parameters, both bactericidal capacity and stress parameters like plasma corticosterone, fecal corticosterone, and cell counts varied significantly by site and individual; however, no ibis sampled from natural sites were included in this study. Bacterial killing assays were performed on plasma samples from American white ibis captured in Palm Beach County, FL in the post-breeding season (fall) and pre-breeding season (spring) prior to this current study, using *Escherichia coli*. Results showed that plasma from ibises captured in urban sites had significantly higher bactericidal capacity than ibis captured in natural sites, contrary to expectation. In the pre-breeding season, plasma from urban ibis killed approximately 75% of E. coli colonies whereas plasma from natural ibis killed approximately ~50% of colonies ($p=.04$). A similar trend occurred in the breeding season ($p=.02$), and when both seasons were combined, the effect grew more significant ($p=<.01$). When examined by site, there was significant variation among both urban and natural sites, reaffirming Curry (2017)'s conclusion that the factors that affect innate immune function vary by site.

In an attempt to better understand the effect of urbanization, particularly anthropogenic resource provisioning, on ibis stress physiology and immune function, we expanded upon this data and Curry's 2016 study. Using the proportion of diet that came from provisioned resources (anthropogenic and refuse) for each individual from Murray et al. (2018), we assessed the effects of urban resource provisioning on stress and immune function, and, unlike Curry 2016, were able to include ibis captured in both urban and natural habitats. In addition to BKAs using E. coli, we also performed BKAs using a relevant *Salmonella* serotype found in ibis to further assess innate immunity. Further, we maintained a captive colony of ibis nestlings and performed a diet trial to specifically assess the effects of the quality of provisioned resources on stress physiology and immune function in white ibis; half of our nestlings were maintained on a diet formulated to mimic a natural diet while the other half received an anthropogenic diet treatment formulated to mimic the resources they receive in urban habitats. Due to more controlled conditions, this experiment allowed us to further assess immune function through the use of carbon clearance, PHA, and febrile challenges in addition to BKAs with both bacteria. We also expanded our stress assessment to include the analysis of heat shock proteins in the nestlings as well as baseline corticosterone analysis. We also assessed if any of the stress or immune parameters were correlated with each other or any other physiological parameters such as mass, sex, body condition, and age.

In our field studies, we expected increased resource provisioning to be negatively correlated with bactericidal capacity, positively correlated with baseline corticosterone levels, and negatively correlated with corticosterone response, potentially acting as an indicator that urban birds are chronically stressed. We also hypothesized that birds with lower bactericidal capacity would have higher baseline and lower induced corticosterone levels. In the captive study, we hypothesized that, in comparison to the control nestlings, nestlings subjected to the anthropogenic diet treatment would have depressed immunity and increased baseline corticosterone and HSP levels due to the nutritional content of the diet, and that immune measures would be negatively correlated with baseline corticosterone and/or HSP levels.

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CHAPTER 2

ANTHROPOGENIC RESOURCE PROVISIONING IN URBAN HABITATS AND ITS EFFECTS ON INNATE IMMUNITY AND STRESS LEVELS IN AMERICAN WHITE IBIS (*EUDOCIMUS ALBUS*)

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Abstract

Urban habitats present changes for wildlife, including shifts in resource availability, diet composition, and exposure to stressors. As natural habitats continue to degrade in response to urbanization, understanding how wildlife respond to these changes is vital for making well-informed conservation and management decisions. Recently, white ibises have become increasingly common in urban habitats where food sources such as human handouts and refuse can be accessed reliably as an alternative to the depleting abundance of their natural prey. These resources, however, vary in nutritional content from ibis' natural prey and are typically lower in protein and higher in simple carbohydrates. Furthermore, the habitats in which these resources are acquired may present novel or additional stressors for ibis, including (but not limited to) aggregations of other wildlife and human disturbance and/or conflict. As a newly urbanized species, we are interested in how ibis physiology responds to these changes in diet and stressors and how this may dictate their potential for success or failure in urban habitats. Thus, we assessed how innate immunity, baseline stress levels, and the stress response vary by the habitat (urban versus natural) ibis were captured in and by the amount of provisioned resources an individual consumes. We found that birds captured from urban sites had higher bactericidal capacity against E. coli than those captured in natural sites. Adults captured in urban habitats also had a significantly lower stress response than adults captured from natural sites; this trend was not present in juveniles. There was a positive relationship between bactericidal capacity against E. coli and the amount of provisioned resources consumed, meaning birds eating more provisioned resources had better innate immunity. There was a negative relationship between resource provisioning and baseline corticosterone in the non-breeding season and a positive relationship between resource provisioning and corticosterone response in the breeding season. Together, these results indicate that urban habitats and the resources provided therein are impacting ibis health, however the implications of these physiological differences remains unclear. Because of the differences in results between habitat and diet comparisons, it's likely that diet is a contributing factor but not the sole factor responsible for physiological changes ibis experience in urban habitats.

Introduction

The ability of a species to adapt to and subsequently succeed in urban environments is an area of study of increasing importance as urbanization continues to transform natural habitats. The effects of urbanization are species-specific, making some species better-equipped for urban habituation than others (Bonier et al. 2007; Evans et al. 2011). This is likely because, central to adapting to a changing environment is the ability to recognize and aptly respond to novel situations or factors—whether , a stressor or a pathogen—without adverse effects on health (Bonier 2012). Supplemental resources in urban habitats provide wildlife with opportunity, however urban habitats are often associated with a suite of changes to which wildlife must adapt if they are to exploit the resources provided there; this is particularly relevant when discussing species of conservation concern and/or those whose natural habitats are disappearing.

In urban habitats, wildlife often receive food in the form of resource provisioning, which can be via human handouts and/or consumption of refuse from sources such as trash cans, dumpsters, and human food that has been left behind. There are many potential stressors associated with resource provisioning in urban environments. Resource provisioning can lead to increased interactions with humans and inter- and intra-species interaction and competition, in addition to attracting wildlife into habitats with environmental stressors such as increased ambient noise and light (Vincze et al. 2016; Lepczyk et al. 2004; McKinney 2006). Under these circumstances, animals may respond so frequently to stressors that they become chronically stressed (Rich & Romero 2005). Chronic stress and constant elevation of circulating stress hormones (e.g. glucocorticoids) has been shown to depress body condition and can result in a cascade of negative physiological effects such as suppression of immune function and inhibition of reproduction (Kleist et al. 2018; Padgett & Glaser 2003; Boonstra et al. 1998; J. Blas et al. 2007). Resources found in urban environments can result in novel aggregations of species or increased population density which may facilitate pathogen transmission. As examples of this idea, there was a greater prevalence of *Mycoplasma* spp. conjunctivitis in house finches gathering at backyard birdfeeders, and in their natural environment, experimentally provisioned raccoons exhibited altered parasite assemblages from those that were not experimentally provisioned (Bradley & Altizer 2007; Hotchkiss et al. 2005; Gompper & Wright 2005). Resources in urban environments, which typically vary in quality and nutrient availability, may also be insufficient to maintain a healthy animal (Becker, Streicker, Altizer, et al. 2015). Many energetically-demanding physiological processes, like immune function, require specific nutrients; for example, T cells require specific protein levels and types to function properly (Murray, Becker, et al. 2016; Lochmiller et al. 1993). As a demonstration of the effects of diet on immune function, bobwhite chicks consuming low-protein diets experienced depressed growth and immunity compared to chicks consuming more protein (Lochmiller et al. 1993). Lack or overabundance of particular micronutrients in provisioned resources may also have severe effects on wildlife health. For example, harp seals fed freshwater smelt and herring, which naturally contains thiaminase, experienced plasma electrolyte imbalances, central nervous system effects, and even death due to thiamin deficiency (Geraci 1972). Finally, supplemental food resources may be contaminated or toxic and can directly impact wildlife health (Henke et al. 2001; Milner et al. 2014).

Many species have adapted to and are still abundant in urban environments, likely because some urban habitats provide supplemental resources for wildlife that are appropriate for healthy function or whose caloric benefits outweigh the costs associated with urban habitats or the quality of resources (Brittingham & Temple 1988). Food availability is the most important factor in determining an animal’s activity budget, thus, the presence of anthropogenic resources may decrease the necessity for extensive foraging, thereby saving time and energy for some species (Orams 2002; Lowry et al. 2013). This benefit may allow allocation of more energy towards energetically-demanding processes such as immune function, self-care, and reproduction, and may ultimately improve overall condition (Houston et al. 2007; Nilsson 2002; Houston et al. 1993). For example, access to anthropogenic resources increased fledgling success for common ravens and banded mongooses with access to supplemental resources carried more fetuses and had higher body condition scores (Webb et al. 2004; Otali & Gilchrist 2004). Appropriate and abundant resources may enhance immune function, as investment in immune function becomes less costly with improved condition and greater access to energy (Møller et al. 1998). To fully exploit anthropogenic resources and urban habitats, some species may adapt to the stressors present in urban environments which could prevent the potential negative effects of a chronically activated stress response on immune function (Atwell et al. 2012).

American white ibises (*Eudocimus albus*) are wading birds commonly found in wetlands along the coast of the southeastern United States. While they continue to breed in mixed-flock rookeries in freshwater wetlands, white ibis have recently become frequent foragers in urban habitats throughout South Florida (Boyle et al. 2014). Florida is one of the most rapidly urbanizing landscapes in the United States, and changes to the landscape have caused wetland degradation driven by anthropogenic factors such as development, contamination, and draining (Chimney & Goforth 2001; Dorn et al. 2011). Likely in response to wetland degradation in Florida, ibis nesting numbers have declined significantly over the past 80 years in the state of Florida, and they are now listed as a Species of Concern (Frederick et al. 2009; Crozier & Gawlik 2003). White ibis naturally prey on aquatic invertebrates, fish, and insects by probing the substrate with their long, decurved bills. Successful utilization of this tactilely-driven foraging mechanism is dependent on specific water levels and soft soil to find prey; therefore, ibis display nomadic behaviors to search for appropriately shallow water in which to forage throughout the year (Bancroft et al. 2002; Kushlan 1986). As a result of recent changes to wetland ecosystems, water levels are often suboptimal for natural foraging and natural prey abundance for white ibis fluctuates more than it has naturally in the past and ibis are now commonly found foraging throughout urban parks, zoos, and residential neighborhoods (Dorn et al. 2011). While they can maintain their natural foraging behavior and consume terrestrial invertebrates and aquatic organisms in these habitats, portions of their diet come directly from human handouts (e.g. park goers tossing bread) or landfills where they forage on anthropogenic food and/or waste (Murray et al. 2018). Previous studies have shown that the provisioned resources ibis consume vary in carbohydrate and protein content from their natural diets and these diets may have implications, both positive and negative, on their physiology and movement patterns (Murray et al. 2018; Kidd 2018).

Foraging in urban habitats not only leads to changes in diet composition but exposes ibis to the aforementioned urban-associated changes, such as shifts in baseline and response stress levels and immune function (Curry 2017). The effects of these shifts are not well-understood, and previous or ongoing studies have shown varying effects of urbanization and provisioned diets on the health and ecology of the American white ibis. Murray et al. (2018) found a negative relationship between urbanization and body condition, likely a result of shifts in dietary quality, as birds captured from more urbanized sites consumed diets higher in urban resources; Hernandez et al. (2016) reported a higher prevalence of *Salmonella* spp. in ibis captured in landscapes affected by urbanization; Curry (2017) found that white ibis spending more time in urban habitats had higher fecal corticosterone levels, indicative of higher baseline stress levels. These results indicate ibis health may respond negatively to urbanization, which has implications for the species conservation and management as well as conservation of their natural habitats. However, Murray et al. (2018) found lower ectoparasite scores in ibis captured in more urban habitats, indicating potential tradeoffs in ibis physiology when foraging in urban areas. In fact, reliable access to supplemental resources (or other urban-associated benefits) may allow urban ibis to commit more time for other activities, such as preening (i.e. to remove ectoparasites). Additionally, Curry (2017) found no effect of increased urban habitat use on plasma corticosterone or innate immune function; however, no ibis from natural habitats were sampled for comparison. To better understand the tradeoffs ibis are presented with in urban habitats and how these tradeoffs affect their physiology and potential for urban habituation, it is imperative to integrate studies of health parameters and ibis behavior and movement.

To better understand the process of physiological acclimatization to urban habitats and the tradeoffs presented therein, we measured how ibis innate immune function, as measured by bacterial killing assays (BKAs) using both *Escherichia coli* and *Salmonella paratyphi B.*, varies by the habitat (urban vs. natural) in which the birds were captured. To take it a step further and test whether resource provisioning may be a contributing factor affecting ibis urban acclimatization, we also analyzed how immune function relates to the proportion of a bird's diet that came from provisioned resources as measured by Murray et al. (2018). We measured baseline corticosterone as well as stress-induced corticosterone (after capture and handling) to assess how urban habitats and anthropogenic resource provisioning affect ibis baseline stress levels and their stress response. Corticosterone is the primary glucocorticoid involved in the avian stress response (e.g. the HPA axis) and has the potential to evolve rapidly (Atwell et al. 2012). Additionally, corticosterone has the potential to both enhance and suppress particular immune system components (Demas et al. 2011). Increased corticosterone levels have been shown to diminish bactericidal capacity, however this response is not consistent. Matson et al. (2006) (five species of tropical birds) and Gao et al. (2017) (house sparrows) found corticosterone depressed immune function as measured by BKAs, Schmidt et al. (2015) (song sparrows) found sex- and bacteria-specific responses, Fratto et al. (2014) found co-infection buffered the depression of bactericidal capacity with elevated corticosterone levels (house finches), and Merrill et al. (2014) found a positive relationship between corticosterone and bactericidal capacity (red-winged blackbirds).

There are several competing hypotheses that we are testing in this experiment. First, ibis may be acclimating well to urban habitats, i.e. habituating to the stressors and/or exploiting the available resources without serious consequences, and thus we would expect urban ibis to exhibit bolstered immune function and lower stress levels. Conversely, urban ibis may be acclimating poorly to urban habitats, and we may see that they have dampened immune function and increased stress levels due to novel pathogen exposure, frequent stressful encounters, a low quality diet, or a combination of factors. Regardless of the hypothesis at play, American white ibis are likely experiencing tradeoffs in urban habitats, and a better understanding of how their physiology is affected by these habitats, particularly when consuming provisioned resources is crucial when making future conservation and management decisions.

Methods

Study Area

To assess how urban resource provisioning affects ibis immune function and stress physiology, we captured ibises at 18 sites within Palm Beach County (PBC), Florida. This is a highly developed county with portions of the Everglades wetland ecosystem located immediately to the west, and white ibis are commonly found in sites throughout PBC. Ten of our sites were initially classified as urban and 8 as natural based on land cover characteristics and the behavior of ibis. In addition to a higher percentage of impervious land cover, urban sites were those where birds were seen being fed or consuming provisioned resources and were habituated enough to human presence to be captured via leg lasso or flip-net. We classified our natural sites based on the lack of habituation to human presence where birds would flush an area if a human came within 30ft (or more). Urban sites included several public parks, a zoo, a landfill, and a residential cul-de-sac. Natural sites consisted of either constructed, restored, or undisturbed wetlands. Details of the sites are summarized in Table 2.1 and Figure 2.1.

Capture and Sample Collection

Ibis were captured during 6 field seasons from 2015-2017. Due to South Florida's tropical climate, the rainy season (May-October) results in significantly more precipitation than the dry season (November-April). Therefore, we captured ibises during three seasons throughout the year representative of the weather fluctuations and ibis life history: pre-breeding (February and March), breeding (June and July), and post-breeding (October and November) (Heath & Frederick 2003). Depending on site type and bird behavior, ibis were either captured using nylon leg lassos or a flip-nets (habituated animals that could be baited) or ibis decoys and mist nets (Hernandez et al. 2016; Heath & Frederick 2003). To obtain a baseline blood sample (time point A; for use in corticosterone and immune assays), birds were physically restrained immediately after capture (while still in net or lasso) for blood collection from the medial metatarsal vein within three minutes of capture to avoid the confounds of handling stress (according to recommendations of Romero and Reed (2005)). Thereafter, birds were held in well-ventilated pillowcases to obtain biological samples, mass, and morphometric measurements. Blood was then collected at two additional time points (Time point B – 15 min. post-capture and Time point C – 30 min. post-capture) from either the metatarsal or jugular vein to assess stress responses provoked by handling of birds; it has been shown in many avian species that this is an adequate amount of time to induce a stress response resulting in an increased concentration of corticosterone in the blood (Romero & Romero 2002). No more than 1% of the bird's mass in total blood volume was collected in a single day. Blood was transferred to heparinized tubes and immediately stored in a cooler with frozen gel packs. Blood samples were centrifuged within two hours of collection for 10 minutes at 3,500 * g to obtain the plasma for our analyses. We then aliquoted 80uL from time points A and B into individual cryovials for use in BKAs and 80uL from time points A, B, and C into individual cryovials for use in corticosterone analyses. Aliquots were stored in liquid nitrogen and at the end of a field season transferred to a -80°C freezer for subsequent analyses, which were conducted within six months of collection.

Bacterial Killings Assays

We used BKAs to measure *in vitro* bactericidal capacity of plasma using an assay that gives a proportion of colony-forming units (CFU) that have been killed by the plasma compared to a control without plasma, following Matson et al. (2006) with minor modifications. Quantification of bactericidal capacity was performed with *Escherichia coli* from a working culture following manufacturer's instructions (*E. coli* ATCC 8739 pellets, Microbiologics, St. Cloud, MN) and *S. paratyphi B*. While *E. coli* is almost exclusively the only bacteria used in BKAs, we included assays using *Salmonella* due to concurrent studies assessing *Salmonella* prevalence and type(s) in American white ibis. Aliquots of *E. coli* and *Salmonella* were made from dilutions of each bacteria with a mixture of 80% culture broth (Sigma-Aldrich Inc, St. Louis, MO) and 20% glycerol (VWR International, Radnor, PA) at a dilution which resulted in ~600 CFUs/10μL when plated and incubated at 37°C. Aliquots were stored in -80°C until use in assays. Ibis plasma was diluted with CO_2-independent media (Life Technologies, Carlsbad, CA) enriched with 4 mM L-glutamine (VWR International). For use in *E. coli* assays, plasma dilutions were optimized to achieve an average of ~30-80% killing using an initial pooled plasma sample resulting in a 1:70 (10μL plasma + 690μL media mixture) dilution of plasma for post-breeding 2015 and pre-breeding 2016, a 1:25 (5μL plasma + 120μL media mixture) plasma dilution for breeding 2016, post-breeding 2016, and pre-breeding 2017, and a 1:10 (15μL plasma + 135μL media mixture) plasma dilution for post-breeding 2017. The variation in plasma dilutions is likely due to the variation in bactericidal capacity by season and year; there were no obvious changes in storage conditions or assay methods that explained this variation. All *Salmonella* assays used a 1:2 (60μL plasma + 60μL media mixture) plasma dilution; no variation in dilution was necessary. 120µL of each plasma dilution was then separately combined with 40µL of each bacterial aliquot (from pre-made aliquots) and left to incubate at room temperature for 30 minutes. 40µL of each bacterial aliquot were separately added to 120µL of CO2-independent media enriched with 4mM L-glutamine (no plasma), as positive controls with ~200 CFUs/50µL. 50µL of all plasma-bacteria combinations and controls were plated onto tryptic soy agar plates (VRW International) in duplicate and spread using a spreading wand which was flame sterilized between each plate. Plates were then incubated at 37°C for 16 hours, at which time plates were removed to count the number of CFUs per plate. The average of the 2 plates per sample, including the controls, was taken; the bactericidal capacity for each sample was then calculated as:

(mean CFU control-mean CFU sample) / mean CFU control

All samples were vortexed prior to and following the addition of a substance and prior to plating to ensure adequate mixing and to prevent bacterial clumping. Bactericidal capacity was primarily assessed using time point A plasma; however, some A samples did not contain enough plasma for both *Salmonella* and *E. coli* assays, so B results were reported for *E. coli*. (Curry 2017 showed no significant difference in the BKA of the two time points for American white ibis and results for which we had both samples confirmed this conclusion). Samples from our first two field seasons were determined to be inactive at the time *Salmonella* BKAs were performed, thus they were excluded from analysis.

Plasma Corticosterone Analysis

Plasma corticosterone was measured at the three time points (A, B, and C) using an enzyme-linked immunosorbent assay (ELISA; Corticosterone ELISA Kit, Enzo Life Sciences) (Herring et al. 2011). Kit instructions for a small sample volume of serum/plasma were followed, resulting in a 1:40 dilution of each plasma sample. The plate reader was blanked against the Blank Wells and optical density of each well was read at 405nm. Corticosterone Standard wells ranged from 32pg/mL to 20,000pg/mL; standards were used to calculate a standard curve on which sample concentrations of corticosterone were calculated using each sample's mean optical density. Time point A was regarded as each individual's baseline. Because the highest corticosterone concentration was found at Time point B for some birds and

Time point C for others, the peak corticosterone response was then calculated as the baseline subtracted from the highest corticosterone concentration (either time point B sample or time point C sample). For individual's with either no time point B or no time point C sample, peak was calculated using the time point available. For individuals with only a time point A sample, only baseline corticosterone was assessed. For individuals missing the time point A sample, no assessment of plasma corticosterone was performed. Once all available samples were taken into account, baseline corticosterone and corticosterone response were assessed (n=158 and n=154, respectively).

Diet Analysis

Provisioned diet was defined as the combination of the diet that came from anthropogenic resources at parks (handouts) and refuse (at landfills), as determined by stable isotope analyses from red blood cells (Murray et al. (2018). Briefly, red blood cells were lyophilized and the carbon and nitrogen isotopic ratios were determined using spectrophotometry. Based on isotopic similarity, five categories of food items were compiled and identified in the samples, two of which were provisioned anthropogenic food (e.g. bread and corn chips) and refuse, which are the data we used in our analyses.

Statistical Analyses

To allow for integration of all plasma dilutions used in *E. coli* BKAs (1:70, 1:25, 1:10) into one analysis, we converted initial *E. coli* BKA values to a uniform scale by multiplying the proportion of colonies killed from each sample by the dilution factor used over the maximum dilution factor used (70) (equation below). This transformed BKA values from assays using a 1:25 or 1:10 dilution to a value calculated on a 1:70 scale (e.g. how many colonies the sample would kill using a 1:70 dilution).

$$\textit{Transformed bactericidal capacity} = \textit{proportion of colonies killed} \times (X^*/70)$$

* X=dilution factor used in BKA (either 70, 25, or 10)

Independent variables were transformed to meet assumptions (R 3.4.1, package: gvlma) (Pena & Slate 2014). This resulted in an arcsine transformation of BKA values and a log transformation of both corticosterone measures.

We then used separate linear models to explore the relationship between response variables (*E. coli* BKA, *Salmonella* spp. BKA, baseline corticosterone, and corticosterone response) habitat and response variables and the proportion of provisioned food in the diet in independent models. We also included year, season, age, and sex, and all 2-way interactions as main effects in our models. Backwards selection by AIC was performed to determine the final model for each response variable against both habitat and diet (R 3.4.1; package: MASS) (Venables & Ripley 2002). Prior to fitting the model, we checked for multicollinearity and excluded any highly correlated variables. We elected to perform a stepwise selection as opposed to candidate model comparison because all variables were of interest or have previously been reported to influence innate immunity. Separate linear regressions and Welch's t-tests were used to quantify the relationships between BKA, baseline corticosterone, and corticosterone response, as well as significant relationships provided in the models. A summary of all model outputs can be found in Table 2.2.

Results

Summary statistics

We captured 170 ibis at 18 sites (average per site=9; range 1-19). We captured the most birds in the post-breeding seasons (66/170) compared to the pre-breeding (59/170) and breeding seasons (45/170). We captured more females than males (106/170) and more adults than juveniles (111/170).

Salmonella BKA

The final habitat model (R^2=0.16, $F_{10,94}$=2.925), which included habitat, season, sex, age, and the following interactions: habitat*season, habitat*sex, and season*age, showed a significant interaction between habitat and season (breeding). Despite this, independent Welch's t-tests used to elucidate this relationship showed no significant effects of habitat on *Salmonella* BKA in any season or when all were combined (Figure 2.3). Similarly, diet had no effect on *Salmonella* BKA according to the final model, which also included season, age, and the interaction between diet and age (R^2=.107, $F_{5,98}$=3.46; Figure 2.2).

E. coli BKA

According to the final habitat model (R^2=.61, $F_{10,161}$=27.93; Table 2.2)—which included habitat, season, age, year, and the following interactions: habitat*season, season*year, and age*year—and subsequent t-tests, birds captured from urban sites had higher bactericidal capacity than birds captured from natural sites in all seasons individually and when all were combined ($t_{43.9}$=-4.35, p=<.001; Figure 2.2). The final diet model for *E. coli* BKA included proportion of diet provisioned, season, sex, age, year, and the following interactions: season*sex, season*year, and age*year (R^2=.51, $F_{11,158}$=17.49; Table 2.2). According to the model, there was a significant positive effect of diet on bactericidal capacity against *E. coli* across all seasons (ß=.389, p=<.02; Figure 2.2).

Baseline Corticosterone

The final habitat model (R^2=.169, $F_{8,151}$=5.03; Table 2.2) included habitat, season, age, and the following interactions: habitat*season and season*age. According to the final model and subsequent t-tests, there was no significant effect of habitat on baseline corticosterone in any season or when all seasons were combined (Figure 2.5). The final diet model for baseline corticosterone included proportion of diet provisioned, season, sex, age, and the following interactions: diet*season, diet*sex, season*sex, and season*age (R^2=.19, $F_{12,145}$=4.19; Figure 2.2). There was a significant negative effect of diet on baseline corticosterone for birds captured in the post-breeding season (ß=-2.2, p=<.003; Figure 2.4). There was a significant interaction between diet and season (breeding), however, a subsequent linear regression of just the birds captured in the breeding seasons showed us this relationship alone was not significant ($F_{4,39}$=3.95, p=.57; Figure 2.4). The interaction between diet and season (pre-breeding) was nearly significantly different from post-breeding (Intercept), and a separate regression of only the birds captured in the pre-breeding season showed the relationship between resource provisioning and baseline corticosterone in the pre-breeding season was insignificant ($F_{4,48}$=.60, p=.98; Figure 2.4).

Corticosterone Response

The final habitat model (R^2=.14, $F_{13,142}$=2.95) included habitat, season, sex, age, year, and the following interactions: habitat*sex, habitat*age, habitat*year, season*year, sex*year, and age*year. According to the final model and t-tests, adult birds captured from urban sites had a significantly dampened corticosterone response compared to adult birds captured from natural sites ($t_{76.65}$=2.79, p=<.01; Figure 2.5), but this relationship was not present in juveniles or when both ages were combined. The final diet model for response corticosterone included proportion of diet provisioned, season, sex, age, and year, and the following interactions: diet*season, sex*year, and age*year (R^2=.11, $F_{10,143}$=3.01; Figure 2.3). According to the model, there was no effect of resource provisioning on corticosterone response. There was a significant interaction between diet and season(breeding), meaning the slope of the relationship was significantly more positive than the slope in the post-breeding seasons (ß=1.6, p=<.04). Separate regressions confirmed there was no significant relationship between diet and response corticosterone in the post-breeding season ($F_{6,50}$=1.31, p=.67) or pre-breeding season ($F_{6,46}$=2.29, p=.26). A separate regression confirmed there was a significant positive relationship between provisioned diet and response corticosterone in the breeding season ($F_{6,37}$=1.64, ß=2.2, p=<.02).

Relationships Between Dependent Variables

There was no relationship between bactericidal capacity (with either bacteria) and baseline corticosterone or corticosterone response. Plasma samples with higher *Salmonella* bactericidal capacity also had higher *E. coli* bactericidal capacity (Adj-R^2=.049, $F_{1,103}$=6.374, p=<.02). Birds with higher baseline corticosterone levels had a greater corticosterone response (Adj-R^2=.035, $F_{1,154}$=6.676, p=<.02).

Discussion

In this study, we assessed the relationship between urban habitats and anthropogenic resource provisioning with innate immune function and stress physiology in a recently urbanized wading bird, the American white ibis. When analyzing by habitat type, we found that birds captured from urban sites had significantly higher bactericidal capacity when challenged with *E. coli*. When challenged with *S. paratyphi B*, there was no difference in bactericidal capacity between birds captured from urban sites and those from natural sites. We obtained similar BKA results when analyzing by the amount of provisioned resources in the diet. As the proportion of the diet that came from provisioned resources increased, we saw an increase in bactericidal capacity when blood was challenged with *E. coli*; this effect was not noted in the *Salmonella* challenges. Though habitat had no effect on baseline plasma corticosterone, we found that adult birds captured from urban sites had significantly weaker corticosterone responses. However, when juveniles were also included in the analyses, this effect disappeared. We also found a negative relationship between resource provisioning and baseline corticosterone in the post-breeding season and a positive relationship between resource provisioning and corticosterone response in the breeding season. In conjunction with results from concurrent studies like Murray et al. (2018), our results indicate that white ibis are likely being presented with physiological tradeoffs in urban habitats and that resource accessibility versus quality could be a driving factor of these tradeoffs. Our results also support the hypothesis that changes to diet in urban habitats are likely contributing to the changes in physiology seen in urban birds, however this is almost certainly not the only factor at play.

Innate Immunity

In contrast to our *a priori* predictions, birds captured in urban habitats killed significantly more *E. coli* colonies in all seasons. Additionally, anthropogenic resource provisioning had a positive effect on innate immune function as measured by BKAs using *E. coli*. Murray et al. (2018) found that ibis consuming larger proportions of provisioned food had diets lower in protein, and their body conditions decreased as provisioning increased. These results led us to hypothesize that bactericidal capacity would decrease as resource provisioning increased, however we did not find this relationship. It is possible that the resources ibis are consuming are quantitatively and qualitatively sufficient to not only maintain a healthy animal but bolster immune function. While incomplete diets have been shown to lead to immunosuppression in mammals and birds, this is typically documented in the adaptive arm of the immune system, where T-cells are dependent on appropriate protein types and sufficient protein levels for proper functioning (Alonso-Alvarez & Tella 2001; Taylor et al. 2013). Though innate immune function in amphibians has been shown to decrease with low dietary protein, ibis innate immune function may not be as dependent on protein or, more likely, may require more drastic changes to protein content before negative effects are seen (Venesky et al. 2012). Murray et al. (2018) showed that ibises captured from sites surrounded by more urban land cover assimilated less δ^{15}N, a proxy for dietary protein, via stable isotope analysis; while these results are significant, the exact protein reduction required to induce changes in ibis physiology is unknown. It is also possible the increased quantity of urban resources (e.g. the caloric benefit) positively impacts innate immune function more than the low quality of urban resources impairs it. French et al. (2008) documented a similar positive effect of urbanization on innate immunity in tree lizards and attributed this effect to access to urban food/water resources. However, bacterial killing assays only test the complement system of innate immune function and do not assess other aspects of innate immunity or any aspect of the adaptive arm of the immune system, so resource provisioning may impact other aspects of immune function that are not represented here. Alternative methodologies, like antibody production against sheep red blood cells, might help to understand these differences but these techniques are difficult to conduct in the field.

Neither habitat nor resource provisioning had a significant effect on bactericidal capacity when challenged with *Salmonella*; however, bactericidal capacity against *Salmonella* was much lower than against *E. coli*, considering the highly concentrated plasma samples necessary to kill even 200 colony forming units of *Salmonella*. Typically, BKAs have exclusively assayed using *E. coli* due to its non-pathogenicity, ease of use in the lab, and accessibility (Demas et al. 2011). We chose to perform our BKAs using both *E. coli* and *Salmonella* due to the relevance of *Salmonella* in this study system as there are several concurrent studies assessing the prevalence and type(s) of *Salmonella* in white ibis, particularly because of their proximity to human and livestock populations. We did see a similar trend between resource provisioning and bactericidal capacity in our *Salmonella* BKA results as in our *E. coli* BKA results, however this trend was insignificant in the *Salmonella* results. We had a smaller sample size for our *Salmonella* assays and could not further concentrate our plasma, so it is possible that with perfected plasma dilutions and more samples, future studies can better elucidate this effect.

Stress Physiology

Our baseline plasma corticosterone values (mean±SE=5.92ng/mL±.45) were comparable to previously recorded values in white ibis by Herring et al. (2011) (5ng/mL±1), slightly higher than values reported by Curry (2017) (1.51ng/mL±1.17), and lower than values reported by Herring et al. (2009) (24.21ng/mL±20.21). We saw no differences in baseline corticosterone between birds captured at urban sites and birds captured at natural sites in any season, consistent with several other species (e.g. European blackbird, Abert's towhee, and dark-eyed junco) (Partecke et al. 2006; Fokidis et al. 2009; Atwell et al. 2012). On the other hand, some studies have found seasonal differences in baseline corticosterone when comparing urban and natural birds. For example, Fokidis et al. (2009) found that urban northern mockingbirds had lower baseline corticosterone levels than birds in natural habitats during the non-breeding season, but this effect disappeared during breeding and post-breeding/molting seasons, consistent with some of our results. Similarly, we found that baseline plasma corticosterone concentrations in ibis significantly decreased as resource provisioning increased for birds captured in the post-breeding season, which is also when ibis molt. Pre-breeding and breeding are typically the most habitat-restrictive seasons for ibis, making the post-breeding season a unique season with fewer habitat restrictions (A. Kidd, unpublished). Murray et al. (2018) determined ibis are most dependent on provisioned resources in the post-breeding season, which may be why we were only able to detect a significant effect of diet on corticosterone levels during this season. Herring et al. (2011) found that during a year of poor food availability, American white ibis nestlings sampled during the breeding season had increased plasma and fecal corticosterone levels, supporting our hypothesis driven by our diet results that low diet quantity and/or quality can increase ibis baseline stress levels.

Traditionally, high baseline levels of corticosterone have been used to identify chronically stressed or unfit individuals, referred to as the "corticosterone-fitness hypothesis" (Romero & Wikelski 2001; Bonier et al. 2009; Breuner et al. 2008). If this is the case, our results might suggest birds consuming higher proportions of provisioned resources in the post-breeding season are healthier or more fit than those consuming lower proportions. In fact, the negative relationship between ectoparasite scores and provisioned diet that Murray et al. (2018) found potentially supports this theory. Other studies have shown that a lack of natural resources may lead to chronic stress and high baseline corticosterone levels for individuals in natural habitats (Hinam & St. Clair 2008; Kitaysky et al. 1999; Bonier 2012). However, we obtained differing results when analyzing by habitat type. Because baseline corticosterone is representative of baseline stress levels in animals, it is therefore easily influenced by the experience of the individual and its perception of stressors. Urban habitats provide a suite of changes aside from diet quality and quantity, and the stressors wildlife encounter likely play a larger role in their stress physiology than just diet alone; this may explain why we got different results when analyzing by habitat versus diet.

There was no significant difference in the corticosterone response between birds captured at natural sites and birds captured at urban sites. However, when juveniles were excluded, adult ibis captured at urban sites had a significantly lower corticosterone response than adult ibis captured at natural sites. There is evidence that the development of the HPA axis response is gradual in altricial and semi-altricial species like the white ibis, which may explain this age difference (Julio Blas et al. 2006). If the juvenile stress response had yet to fully develop at the time of sampling, it would be difficult to identify any patterns based on habitat. Additionally, juveniles likely lack exposure to the multitude of stressors that adults have experienced, so their responses may not be individualized yet as is possible in adults (i.e. changes to the perception of a stressor). Based on these results and the positive relationship between urbanization and anthropogenic resource provisioning, we expected to see a similar relationship in our diet results: that is, as resource provisioning in the diet increased, corticosterone response would decrease, at least in adults. However, when analyzed by diet, we found that corticosterone response significantly increased with resource provisioning in the post-breeding season. This effect was similar to the effect French et al. (2008) saw in house sparrows. Although from rural sites, house sparrows with access to abundant supplemental resources from nearby farms showed strong corticosterone responses during the breeding season. These, along with our results, may suggest that diet is an influential factor in stress responsiveness but its effects differ from the effects of habitat alone. It has been hypothesized that consistent access to resources may prevent chronic stress (Goymann & Wingfield 2004). While many birds in unpredictable habitats suppress their stress response during breeding to prevent deleterious energy expenditure and behaviors that may affect reproduction, the predictability of urban resources may act as a buffer to this effect (J. C. Wingfield & Sapolsky 2003). However, this hypothesis has yet to be tested, and the down-regulation of the HPA axis caused by unpredictable habitats during breeding has not been explicitly documented in ibis.

There are competing hypotheses regarding how to interpret the corticosterone response. An attenuated response, in conjunction with high baseline levels, has been thought to indicate a chronically stressed animal (Rich & Romero 2005). If this is the case for ibis, it may support the hypothesis that ibis consuming more provisioned resources are fitter than those consuming smaller amounts, perhaps priming the species for success in urban habitats. In contrast, given our habitat results and their dampened corticosterone response, it may mean that adult ibis captured from urban habitats may be chronically stressed. However, we did not find concurrent, significantly higher baseline corticosterone levels in adult urban ibis (Rich & Romero 2005). On the other hand, an attenuated response could potentially indicate better habituation to anthropogenic stressors, therefore responding less dramatically or frequently, which has been recorded in other species (e.g. Magellanic penguins) (Brian G Walker et al. 2006). This hypothesis could mean birds consuming greater proportions of provisioned resources are experiencing frequent stressors to which they have yet to habituate, as their response was significantly greater. It could also mean that adult ibis captured from urban sites are better habituated to the stress of urban life considering their dampened response. Due to the contrasting and difficult-to-interpret hypotheses, more research into ibis stress physiology is necessary. Ideally, future studies would involve resampling and include more measures of physiological stress, such as heat shock proteins, fecal and/or feather corticosterone levels, and heterophil/lymphocyte ratios, which would provide us with better information on how ibis are adapting over time to urban life and/or provisioned diets (Herring & Gawlik 2007; Sheriff et al. 2011; Davis et al. 2008).

In conclusion, American white ibis physiology may be affected both by urban habitats and the change in diet they are experiencing due to degradation of natural habitats and prey abundance. Our immune function assay results indicate that despite previously recorded declines in body condition by Murray et al. (2018), ibis innate immunity against *E. coli* is bolstered in urban habitats and as they consume greater proportions of provisioned resources, indicating a potential health trade-off. We saw competing results in regards to baseline corticosterone and corticosterone response when analyzing by habitat versus diet, but due to the lack of evidence characteristic of chronically stressed animals and the absence of a negative relationship between stress parameters and innate immunity, we did not find that corticosterone levels or responses provided evidence for urban ibis experiencing chronic stress in urban habitats/with increased resource provisioning. However, the competing hypotheses concerning how to interpret corticosterone results require further studies into this species' stress physiology, and additional and more extensive sampling and assaying would provide a better indicator of their health as they continue to forage in urban habitats and on provisioned resources.

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Tables and figures

Table 2.1. Summary of capture sites. N: total sample size per site. Avg PD: Average proportion of diet consisting of provisioned foods. By-site averages for each response variable: S-BKA (bactericidal capacity using *Salmonella*), E-BKA (bactericidal capacity using *E. coli*), PCORT-A (baseline plasma corticosterone), and PCORT-R (corticosterone response; peak corticosterone-baseline corticosterone) as well as the sample size for each variable by site.

Site (Code)	N	Avg. PD	Hab. Type	Response Variable(N)			
				S-BKA	**E-BKA**	**PCORT-A**	**PCORT-R**
Busch Wildlife Sanctuary (BWS)	1	0.54	Natural	0.65(1)	0(1)	NA(0)	NA(0)
Cat cul-de-sac (CAT)	2	0.63	Urban	0.26(2)	0.13(2)	3.62(2)	31.22(2)
Dreher Park (DH)	13	0.56	Urban	0.21(10)	0.38(13)	7.8(12)	38.09(12)
Dubois Park (DB)	7	0.72	Urban	NA(0)	0.82(7)	8.24(5)	41.55(5)
Gaines Park (GP)	14	0.57	Urban	.17(10)	.44(14)	5.61(14)	33.94(13)
Indian Creek Park (IC)	17	0.57	Urban	.20(10)	.35(17)	4(16)	42.22(16)
Juno Beach Park (JB)	12	0.72	Urban	.17(6)	.48(12)	5.95(9)	50.00(9)
JW Corbett (JWC)	8	0.46	Natural	.09(7)	.11(8)	7.34(7)	66.44(7)
Kitching Creek (KC)	7	0.55	Natural	NA(0)	.53(7)	10.44(7)	34.59(7)
Lake Worth (LKW)	5	0.54	Urban	.17(5)	.22(5)	3.40(5)	33.21(5)
Lion Country Safari (LCS)	16	0.54	Urban	.19(9)	.54(16)	4.42(15)	38.91(13)
Loxahatchee Slough (LSL)	7	0.42	Natural	0.05(7)	.01(7)	4.72(7)	59.19(7)
Loxahatchee Wildlife Refuge (LWR)	7	0.35	Natural	NA(0)	.42(7)	10.24(6)	24.42(5)
LWR-Northeast Side (LWRNE)	6	0.47	Natural	NA(0)	.58(6)	10.13(6)	54.10(6)
Royal Palms Park (RP)	2	0.50	Urban	.17(2)	.25(2)	2.28(2)	69.68(2)
Solid Waste Authority (SWA)	12	0.60	Urban	.27(9)	.36(13)	7.76(12)	42.50(12)
Tetra Tech Mitigation Bank (TT)	8	0.42	Natural	.08(2)	.43(8)	8.05(8)	44.54(8)
Wakodahatchee Wetlands (GC)	26	0.63	Natural	.23(24)	.07(26)	3.10(26)	41.36(26)

Table 2.2. Summary of model outputs for each response variable tested in American white ibis with *the proportion of provisioned resources in the diet* included in the model as the primary explanatory variable of interest on the left and *habitat* included in the model as the primary explanatory variable of interest on the right. Estimate, t value, and p value for each effect included in the final models. Only significant effects are included. The descriptor for each (Intercept) term can be found in the results section. Cort=corticosterone. PD=proportion of diet consisting of provisioned foods. N=sample size for each model.

Resource Provisioning

Response variable	**Effect**	**Estimate**	**t**	**p**
BKA (*Salmonella*)	(Intercept)	0.20	1.33	0.185
N=104	PD	0.40	1.50	0.136
	Season(breeding)	-0.088	-1.30	0.198
	Season(post-breeding)	0.091	1.45	0.151
	Age(juvenile)	0.22	1.02	0.312
	PD*age(juvenile)	-0.58	-1.52	0.132
BKA (*E. coli*)	(Intercept)	0.75	6.11	7.53e-9
N=170	PD	0.39	2.41	0.017
	Season(post-breeding)	-0.50	-5.24	5.14e-7
	Age(juvenile)	-0.24	-3.17	0.0019
	Year(2)	-0.61	-7.03	6.03e-11
	Season(breeding)*sex(male)	-0.29	-2.54	0.012
	Season(post-breeding)*year(2)	0.47	3.87	1.6e-4
Baseline cort	(Intercept)	2.94	6.55	9.16e-10
N=158	PD	-2.21	-3.09	0.0024
	Season(post-breeding)	-2.38	-3.34	0.0011
	Sex(male)	-1.04	-2.07	0.040
	PD*season(post-breeding)	3.07	2.82	0.006
	Season(post-breeding)*sex(male)	0.91	2.81	0.0057
	Season(breeding)*age(juvenile)	0.50	1.66	0.0997
Cort response	(Intercept)	3.21	11.90	<2e-16
N=156	Season(breeding)	0.78	2.11	0.037
	Season(post-breeding)	-0.93	-2.01	0.046
	Sex(male)	0.36	2.50	0.014
	Age(juvenile)	-0.28	-1.99	0.049
	PD*season(post-breeding)	1.63	2.13	0.035
	Age(juvenile)*year(2)	0.42	2.25	0.026

Habitat

Response variable	**Effect**	**Estimate**	**t**	**p**
BKA (*Salmonella*)	(Intercept)	0.178	1.42	0.159
N=105	Season(post-breeding)	0.434	3.156	0.0022
	Habitat(urban)*season(post-breeding)	-0.291	-2.12	0.037
	Season(breeding)*age(juvenile)	-0.337	-2.191	0.031
	Season(post-breeding)*age(juvenile)	-0.318	-2.474	0.015
BKA (*E. coli*)	(Intercept)	0.827	12.82	<.0001
N=172	Habitat(urban)	0.334	4.714	<.0001
	Season(post-breeding)	-0.370	-3.521	0.0006
	Age(juvenile)	-0.179	-2.671	0.008
	Year(2)	-0.696	-9.30	<.0001
	Habitat(urban)*season(post-breeding)	-0.223	-2.026	0.044
	Season(post-breeding)*year(2)	0.497	4.679	6e-06
	Age(juvenile)*year(2)	0.223	2.477	0.0143
Baseline cort	(Intercept)	1.9215	9.856	2e-16
N=160	Season(post-breeding)	-0.850	-3.062	0.0045
	Habitat(urban)*season(breeding)	0.693	2.256	0.031
	Habitat(urban)*season(post-breeding)	0.873	2.84	0.0079
Cort response	(Intercept)	3.573	25.03	2e-16
N=156	Habitat(urban)	-0.349	-2.291	0.0235
	Age(juvenile)	-0.559	-3.388	0.0009
	Habitat(urban)*age(juvenile)	0.626	3.165	0.0019
	Sex*year(2)	-0.555	-2.981	0.0034

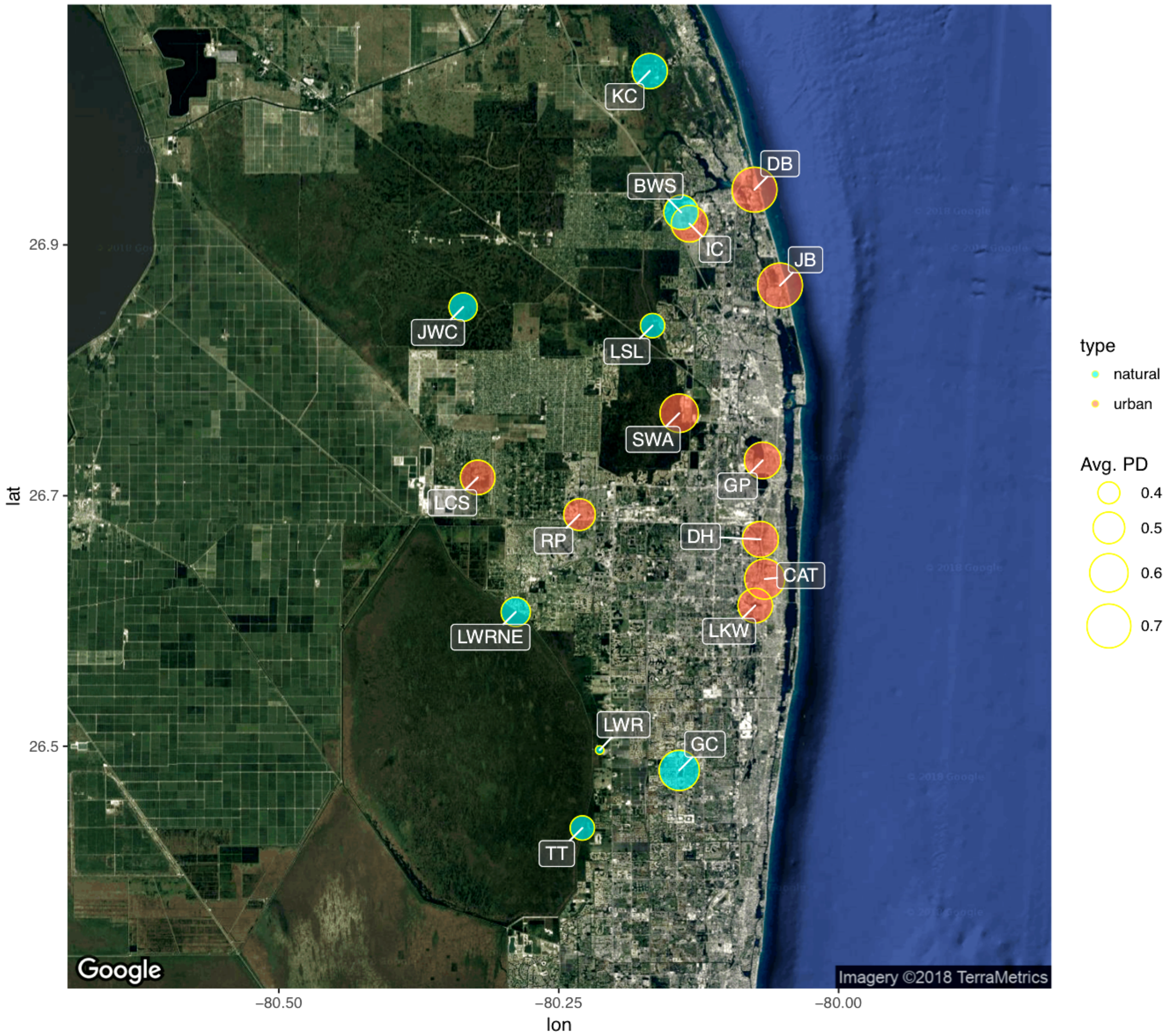

Figure 2.1. Map of the locations where American white ibis were captured in Palm Beach County (PBC), Florida. Size of points are scaled by the site average of proportion of diet that was provisioned (Avg. PD) as determined by stable isotope analysis (Murray et al. 2018). Green circles represent natural sites. Orange circles represent urban sites. Sites are labeled by site codes. (see Table 1).

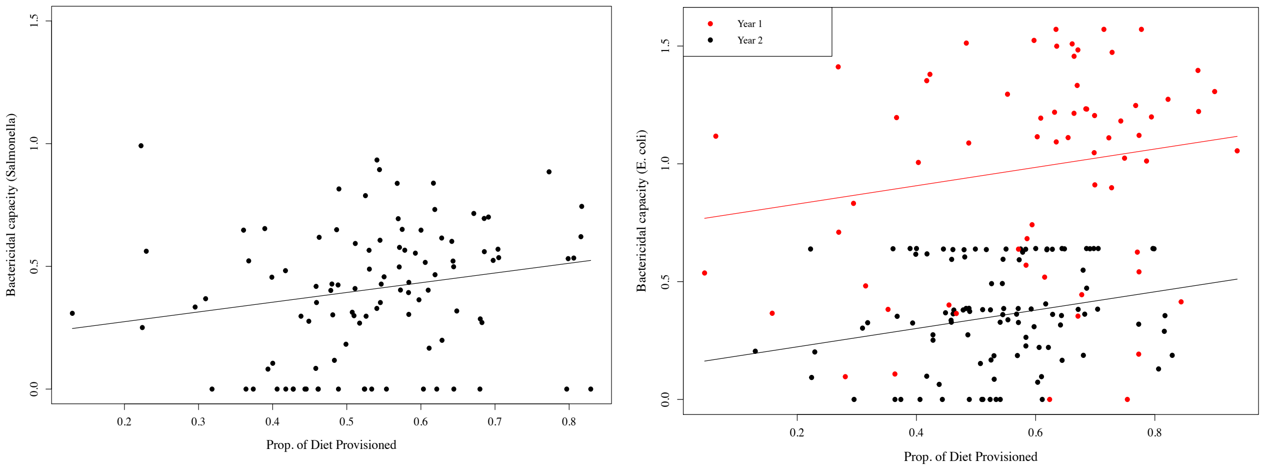

Figure 2.2. Left. Increased resource provisioning (as determined by stable isotope analysis) had no significant effect on bactericidal capacity in American white ibis when ibis plasma was challenged with *Salmonella*. **Right.** Resource provisioning had a significant positive effect on bactericidal capacity in American white ibis in both year 1 (red) and year 2 (black) of sample collection when challenged with *E. coli*. Samples collected in year 1 killed significantly more *E. coli* colonies than year 2 samples (intercepts), however the effect of resource provisioning (slope) was the same in both years. **Note**: Y-axis denotes transformed values.

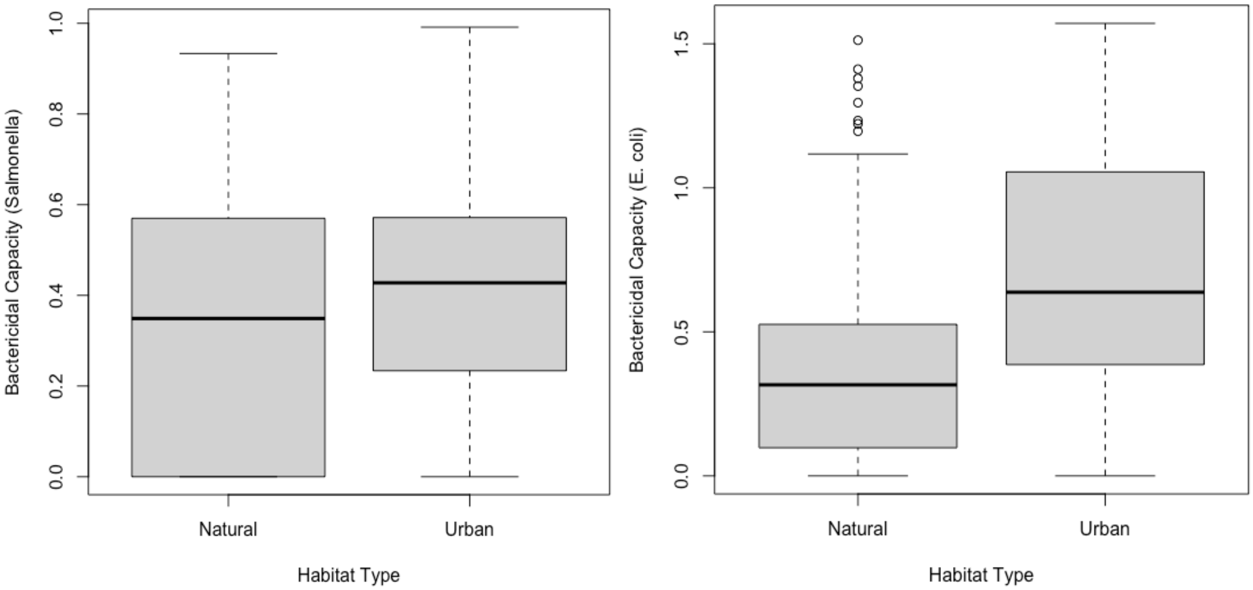

Figure 2.3. Left. American white ibis captured in urban habitats in Palm Beach County, FL had similar bactericidal capacity as birds captured from natural habitats when challenged with *S. paratyphi B*. **Right**. Ibis captured from urban habitats killed significantly more *E. coli* colonies than birds captured from natural habitats. This trend was also evident when split out by season (not pictured).

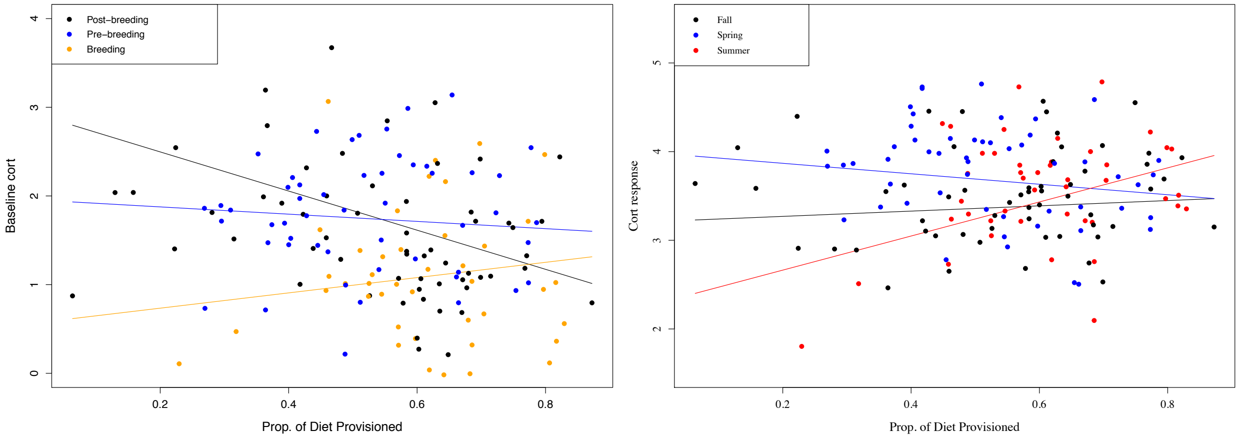

Figure 2.4. Left. As resource provisioning increased, American white ibis had significantly lower baseline plasma corticosterone in their post-breeding season (black). This relationship was not evident in either the pre-breeding (blue) or breeding (red) seasons. **Right.** As resource provisioning increased, the corticosterone response (as calculated by peak minus baseline) of white ibis increased in the breeding season (red). The effect of diet was not significant in post- (black) or pre-breeding (blue) seasons. Proportion of diet that was provisioned was calculated using stable isotope analysis.

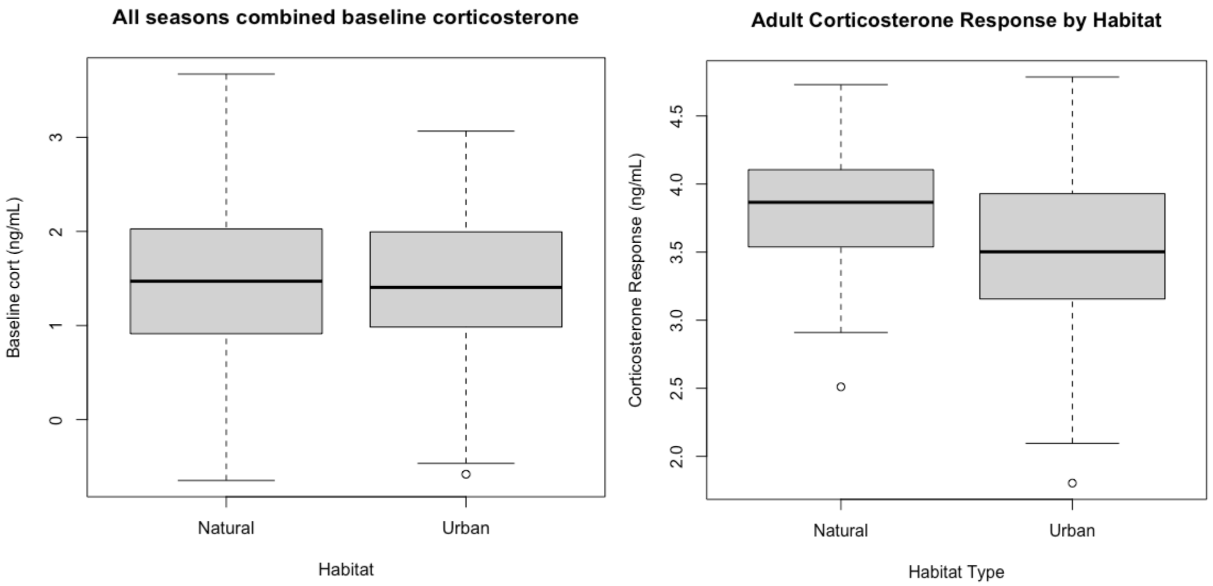

Figure 2.5. Left. White ibis captured from urban and natural habitats in Palm Beach County, FL had similar baseline plasma corticosterone responses when all seasons (pre-, post-, and breeding) were combined (pictured) or when split out by season (not pictured). **Right**. White ibis adults captured from natural sites had a significantly higher corticosterone response than those captured from urban sites. This effect was not seen in juveniles (not pictured) or when both ages were combined (not pictured).

CHAPTER 3

THE EFFECTS OF AN ANTHROPOGENIC DIET ON STRESS AND IMMUNITY IN AMERICAN WHITE IBIS (*EUDOCIMUS ALBUS*) NESTLINGS RAISED IN CAPTIVITY

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Abstract

When wildlife transition into urban habitats, they often experience a shift in resource availability and the quality of their diet. While reliable resources may provide benefits for wildlife in the form of readily available energy, the quality of these foods often varies from what they typically consume in natural habitats. This presents potential tradeoffs for wildlife and may affect their physiology both beneficially and adversely. To assess these tradeoffs and determine the effects of an urban-associated shift in diet quality on immunity and stress in a newly urbanized species, the American white ibis, we exposed 10 white ibis nestlings to an experimental anthropogenic diet while 10 were maintained on a natural diet. With the addition of white bread and reduction in seafood content, the 10 nestlings in the anthropogenic group consumed a diet representative of what they consume in urban habitats: low protein and high carbohydrate content. We assessed innate immunity via bacterial killing assays (BKAs) and an in vitro carbon clearance assay, adaptive immunity via an in vivo phytohemagglutinin assay, and baseline stress levels via plasma corticosterone and heat shock protein 70 levels in the blood. The anthropogenic diet had no effect on any measure of nestling immunity or stress either between or among diet groups. We found a negative relationship between BKA and PHA measures, indicating potential tradeoffs between the adaptive and innate immune systems. The nestlings were only exposed to this diet for 30 days when sampled to assess the effects following the change in diet; this may have not been a long enough period of time to induce those physiological changes. More likely, however, is the fact that in urban habitats, animals are exposed to a suite of changes in addition to the quality of resources. It is possible that a change in quality alone is not enough to induce the physiological changes we have seen in urbanized ibis in the wild.

Introduction

Urban habitats present many changes for wildlife, including a change in the abundance and makeup of resources provided both intentionally (e.g. bread to birds in parks) and unintentionally (e.g. waste from landfills) to wildlife; as a result of resource provisioning, many species experience a shift in the types of foods they consume. For example, urban common ravens consumed more trash than natural counterparts and urban red foxes consumed more scavenged meat, compost, pet food, and berries than peri-urban foxes (Kristan III et al. 2004; Contesse et al. 2004). Consistent and predictable access to provisioned food has the potential to alleviate the effects caused by lack of natural food and nutrient availability, often the most limiting factor for wildlife (Ostfeld & Keesing 2000; Sinclair & Krebs 2002; Orams 2002). Sufficient resource abundance may allow allocation of more energy towards energetically-demanding processes such as immune function, self-care, and reproduction, and may ultimately improve overall condition (Houston et al. 2007; Nilsson 2002; Houston et al. 1993). For example, access to anthropogenic resources increased fledgling success for common ravens, and banded mongooses with access to supplemental resources carried more fetuses and had higher body condition scores (Webb et al. 2004; Otali & Gilchrist 2004). Appropriate and abundant resources may enhance immune function, as investment in immune function becomes less costly with improved condition and greater access to energy (Møller et al. 1998). This has been seen in white ibis captured from urban habitats and those consuming greater proportions of urban-associated resources (Cummings et al., unpublished).

However, there are potential consequences of constantly consuming provisioned resources. Resource provisioning can diversely affect the hormonal response to stress for individuals as they frequently interact with humans and other wildlife. For example, juvenile hoatzins at tourist-exposed sites had a stronger hormonal response to experimental stress than those at undisturbed sites, but through reduced flush distances, adult hoatzins exhibited habituation to tourist disturbance (Müllner et al. 2004). Furthermore, the nutritional content of provisioned resources may differ from natural resources in several ways. For example, urban diets may often lack essential nutrients that are typically provided through natural diets, such as protein. Constantly consuming a suboptimal diet can have detrimental effects on wildlife health, particularly on immunity, as many aspects of immune function require specific nutrients (e.g. T-cells require specific protein levels to properly function) (Cooper et al. 1974). Additionally, energetically insufficient diets can lead to an immune-deficient animal, particularly when investment of available energy is allocated towards other processes, such as growth, reproduction, and ornamentation (Taylor et al. 2013; Lochmiller & Deerenberg 2000; Forbes et al. 2016). Relationships between diet and immunity have been studied in several wild species. For example, bobwhites consuming diets lower in protein had depressed lymphoid function (Lochmiller et al. 1993); urban coyotes consuming greater proportions of urban resources had decreased body condition and greater prevalence of disease, and protein content has been hypothesized as the main factor driving this relationship (Murray, Hill, et al. 2016). Additionally, changes in micronutrient levels in the diet can have implications on wildlife physiology. For example, harp seals fed freshwater smelt and herring, which naturally contains thiaminase, experienced plasma electrolyte imbalances, central nervous system effects, and even death due to thiamin deficiency (Geraci 1972). Moreover, female lesser black-backed gull supplemented with carotenoid-rich food had reduced immunoglobulin levels than non-supplemented birds, indicating suppressed immunity (Blount et al. 2002). However, it was hypothesized that this relationship was actually caused by a reduced need for immunoglobulins due to enhanced efficiency of the innate immune response when provided with supplemental carotenoids. These results demonstrate how changes in diet quality may present tradeoffs within the immune system and that enhanced or depressed immunity is not always indicative of a healthy or unhealthy animal. Elucidating how the physiology of a species is affected by an anthropogenic diet, either beneficially or adversely, and how quality versus quantity tradeoffs play out can help implement relevant conservation programs, dictate management decisions, and provide insight into the relationship between humans and urban wildlife, which is particularly important for species of concern or those whose natural foraging opportunities are limited.

American white ibis (*Eudocimus albus*; family: Threskiornithidae, order: Pelecaniformes) are wading birds commonly found in wetlands along the coast of the southeastern United States. While they continue to breed in mixed-flock rookeries in freshwater wetlands, white ibis have recently become prevalent foraging during the day in urban habitats throughout South Florida (Boyle et al. 2014). Florida is one of the most rapidly urbanizing landscapes in the United States, and changes to the landscape have caused wetland degradation driven by anthropogenic factors such as development, contamination, and draining (Chimney & Goforth 2001; Dorn et al. 2011). In response to wetland degradation, ibis breeding numbers have declined significantly over the past 80 years in the state of Florida, and they are now listed as a Species of Concern (Frederick et al. 2009; Crozier & Gawlik 2003). White ibis naturally prey on aquatic invertebrates, fish, and insects by probing the substrate with their long, decurved bill. Successful utilization of this tactilely-driven foraging mechanism is dependent on specific water levels and soft soil to find prey, which means ibis display nomadic behaviors to search for appropriate foraging areas throughout the year (Bancroft et al. 2002; Kushlan 1986). As a result of recent changes to wetland ecosystems, water levels are often suboptimal for natural foraging and natural prey abundance for white ibis fluctuates more than in the past (Dorn et al. 2011). Ibis are now commonly found foraging throughout urban parks, zoos, and residential neighborhoods, where the bulk of their diet comes directly from human handouts (e.g. park goers tossing bread) and neighborhoods or landfills where they forage on anthropogenic food and/or waste (Murray et al. 2018). They also maintain their natural foraging behavior and consume terrestrial invertebrates and aquatic organisms living in urban water sources; however, some flocks appear to be dependent solely on human handouts and supplemental resources (Welch 2016). Supplemental provisioning also influences ibis site fidelity to urban habitats and their movements (Murray, Hernandez, et al. in press; Kidd 2018).

Curry (2017) found significant variation in the isotopic signatures of ibis diets (i.e. $\delta^{13}C$ and $\delta^{15}N$) as the level of the urbanization of the capture site increased, reflecting changes in ibis diet as habitat changes. Additionally, Murray et al. (2018) showed that ibis that were captured from highly urbanized sites (as determined by surrounding land cover) assimilated more anthropogenic resources and less dietary protein ($\delta^{15}N$). Ibis health in response to anthropogenic resources and synurbanization have been studied in concurrent field studies, and the effects of this shift in habitat and diet composition vary, likely indicating tradeoffs for urban ibis. Murray et al. (2018) found a negative relationship between resource provisioning and body condition, possibly as a result of shifts in dietary quality. These results may suggest ibis health responds negatively to resource provisioning, and by proxy, urbanization, which has implications for its conservation and management. However, Murray et al. (2018) found lower ectoparasite scores in ibis consuming more provisioned resources and we have found that innate immune function was better, and baseline corticosterone decreased when greater proportions of the diet came from provisioned resources (Cummings, unpublished data). Additionally, Cummings et al. (unpublished) found that birds captured from urban habitats had enhanced innate immunity and an attenuated stress response, but interpretations of these results are complicated; enhanced innate immunity does not reflect all aspects of the immune system, which experiences a suite of energetic tradeoffs, and a suppressed stress response may indicated a habituated animal or one who is incapable of mounting an appropriate response due to chronic stress. These studies indicate potential tradeoffs in ibis physiology when foraging in urban areas; however, whether diet quality, diet quantity, or other characteristics of urban habitats are directly responsible for these effects is unknown.

One of the biggest challenges in assessing urban diets and their effects on urban wildlife health is the naturally confounding factors associated with field settings. While urban resources have been shown to both improve and impair fitness, the cause of these effects cannot be definitively assigned, as field or observational studies do not typically allow for isolation and manipulation of just one variable of interest. Additionally, wildlife nutritional studies often require repeated field observations and the best physiological assessments of nutrition require invasive sampling techniques, making robust results hard to obtain (Murray, Becker, et al. 2016; Page & Underwood 2006). To isolate the main effect of diet quality on ibis health, we raised a captive colony of ibis nestlings and subjected half of the nestlings to an anthropogenic diet designed to mimic urban resources in the wild. We performed several immune assays before and after the diet change to determine whether diet quality influenced multiple aspects of their immunity; multiple assays were used to best capture the effects of diet on the diverse arms of the immune system, which may not all respond identically. In addition to their ubiquity in the literature, the assays chosen were also used due to their minimal invasiveness, as these birds were involved in concurrent studies. Because resource provisioning can affect stress levels, and because the immune system and the HPA axis (e.g. physiological stress response) are tightly connected, we also quantified baseline stress levels by measuring circulating levels of corticosterone, as corticosterone has the potential to both enhance and suppress particular immune components (Demas et al. 2011). For example, elevated corticosterone levels have been shown to both diminish (Matson et al. 2006; Gao et al. 2017) and enhance innate immune function (Merrill et al. 2014) measured by bacterial killing assays. We analyzed heat shock protein levels, which function as a longer term measure of stress, as they are released in response to a variety of stressors—including nutritional stress—at a slower rate than corticosterone (see Herring and Gawlik (2007) for a review of the use of stress proteins in avian ecology) (Moreno et al. 2002). Ibis nestlings exposed to a nutritional stressor in the field (food limitation) had both elevated levels of corticosterone in circulation and elevated levels of heat shock protein in the blood, making these measures a relevant investigation into the relationship between diet quality, nutritionally-induced stress, and immunity. We hypothesized that nestlings consuming the anthropogenic diet would show enhanced immune function on chosen immune function assays, decreased baseline corticosterone and heat shock protein levels suggestive of reduced physiological stress, and that there would be a negative relationship between stress levels and immune function. These hypotheses reflect results we found in white ibis in the wild during our concurrent study that suggest anthropogenic resources may be beneficial for white ibis in the wild.

Methods

Nestling husbandry

In April 2017, we collected an experimental colony of 20 free-ranging American white ibis nestlings (ranging from 10-14 days of age) from the 6th Bridge rookery, in Broward County, FL (GPS cords). Upon collection, nestlings were color-banded for individual identification and transported to Athens, Georgia where they were maintained at the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia from April 2017-October 2017. At the PDRC, all birds were initially housed inside a modified chicken house, in a single pen (HxWxL=3.9m x 3.9m x 5.9m). The cement floor was covered with artificial grass matting, and the birds were provided with full-spectrum lights (ZooMed Avian Sun 5.0, San Luis Obispo, CA), perches of multiple sizes and heights, a wading pool, constant clean drinking water in separate bowls, natural and synthetic browse to promote nestling development and provide space for hiding. Initially the room temperature was tightly controlled with heaters to accommodate a nestling's inability to thermoregulate. At admission to PDRC, all birds were fed a piscivore gruel (Emeraid, Cornell, IL, USA) by gavage tube. After 3 days, they were fed by syringe and by day 7, most were transitioned to hand-feeding. At day 21 post-collection, the birds were divided into two groups housed in identical pens by age, such that larger/older birds that were capable of feeding independently off of platters were grouped together and those still requiring syringe feeding formed a second group. By day 93, the sex of all birds had been determined by PCR and all were feeding exclusively from platters, thus birds were randomly assigned to one of two pens to prevent size/age bias while ensuring equal sex ratios within each pen to prevent sex bias (5 females, 5 males; n=10 per pen). These pens would eventually represent the diet treatments the birds received. As the seasons changed, birds were maintained at temperatures representative of ibis natural habitat, ranging from 24-30°C, using heaters, fans, and cooling towers as necessary and constant monitoring of ambient temperature and humidity with environmental thermometers. Each pen was divided into two halves with a plastic curtain, and the birds were gently shuttled to one half while the other was cleaned daily. Artificial grass matting, water bowls, wading pools, browse, and any other enrichment items were taken outside the building and cleaned thoroughly with soap and water, sprayed with 10% bleach solution, rinsed and fully dried (Fowler & Miller 2012). The cleaning schedule rotated so that half of a pen went uncleaned no longer than three days. Once cleaned, materials were put back in the pen and natural browse was replaced. Because American white ibis develop rapidly, the husbandry and enrichment methods were constantly adjusted to meet their needs (Fowler & Miller 2012).

Diet

Ibis nestlings are semiprecocial and develop very rapidly, thus their diet delivery and composition required constant adjustment based on nutritional needs. The diets for both treatment groups were based on the diets of ibis raised in captivity at zoological facilities, but modified to meet nutritional requirements in collaboration with an animal nutritionist (Dr. Lisa Hoopes; Table 4.1). In general, once birds were self-feeding from platters they were consuming a diet composed of nutritionally-balanced commercially-available pellets designed for growing flamingos (Mazuri Flamingo Breeder; St. Louis, MO, USA) to which the following were added: seafood (shrimp and smelt), cooked egg (for additional protein), water, and vitamin/micronutrient supplements. Until day 106, all birds were maintained on a balanced diet representative of components and nutrients the birds would be consuming in their natural habitats. At day 113, one pen was randomly assigned to the anthropogenic diet treatment and gradually received a greater proportion of simple carbohydrates (white bread) while simultaneously the protein content was decreased by reducing the amount of seafood. By day 120, the nestlings fed the anthropogenic diet were consuming a diet consisting of 40% white bread by weight. This diet was designed to mimic diets wild ibis consume in urban habitats, which are typically lower in protein and higher in carbohydrates due to the increase in human handouts and consumption of refuse as opposed to natural foraging of aquatic invertebrates. A summary of the diets can be found in Table 3.1.

Collection of biological samples

For the first few weeks of maintenance, nestlings were handled every day to monitor their growth through obtaining a body mass and standard avian morphometric measurements (tarsus length, tarsus width, and culmen length), and to monitor for abnormalities (such as pododermatitis) or delayed growth. By day 47, nestlings were transitioned to handling every other day, and by day 74, were only handled weekly to obtain a body weight, morphometric measurements or on and as-need basis. Blood was collected from the birds at day 106 for pre-diet BKA and corticosterone, day 143 for post-diet BKA and corticosterone, day 149 for carbon clearance, and day 173 for HSP70 (Figure 4.1). Blood was collected from either the jugular or the medial metatarsal vein using a 25-gauge needle (Becton, Dickinson and Company, Franklin Lakes, NJ). No more than 1% of the bird's body weight in blood volume was collected and birds were never bled more than once in a 7-day period. After collection, blood was transferred into 3 mL heparinized (for BKA, corticosterone, and HSPs) or EDTA (for carbon clearance) vacutainer tubes (Becton, Dickinson and Company) and immediately placed on ice. When collected for use in bactericidal assays and corticosterone analysis, blood was transferred on ice back to the laboratory and centrifuged at 3,500 g for 10 minutes to obtain plasma. Approximately 80 uL of plasma was aliquoted into separate 2 mL cryovials (Corning, Corning, NY), for either BKAs or corticosterone analysis. Cryovials were then immediately placed in -80°C for storage until use in respective assays. The red blood cells remaining after centrifugation were transferred to a separate cryovial, stored -80°C for storage until used for heat shock protein analyses. The *in vitro* carbon clearance assay required 1 mL of whole blood, thus, once collected into EDTA-lined tubes, samples were immediately transferred on ice to the laboratory for analysis. All animal procedures were reviewed and approved by the University of Georgia's Institutional Animal Care and Use Committee (AUP#A2016 09-012).

Innate Immunity—bactericidal capacity

We used bacterial killing assays (BKAs) to measure *in vitro* bactericidal capacity of plasma using an assay that gives a proportion of colony-forming units (CFU) that have been killed by the plasma compared to a control without plasma following Matson et al. (2006) with minor modifications. Quantification of bactericidal capacity was performed with *Escherichia coli* from a working culture following manufacturer's instructions (*E. coli* ATCC 8739 pellets, Microbiologics, St. Cloud, MN) and *Salmonella paratyphi B*. Aliquots (500 µL) of *E. coli* and *Salmonella* were made from dilutions of each bacteria with a mixture of 80% culture broth (Sigma-Aldrich Inc, St. Louis, MO) and 20% glycerol (VWR International, Radnor, PA) at a dilution which resulted in ~600 CFUs/10µL when plated and incubated at 37°C. Aliquots were stored in 1.7mL microcentrifuge tubes (Corning) in -80°C until use for BKAs, ibis plasma was diluted with CO_2-independent media (Life Technologies, Carlsbad, CA) enriched with 4 mM L-glutamine (VWR International). For use in E. coli assays, plasma dilutions were optimized to achieve an average of ~30-80% killing using an initial pooled plasma sample resulting in a 1:15 (10µL plasma + 140µL media mixture) dilution of plasma. For *Salmonella* assays, we used a 1:2 (60µL plasma + 60µL media mixture) plasma dilution. Then, 120µL of each plasma dilution was then separately combined with 40µL of each bacterial aliquot (from pre-made aliquots) and left to incubate at room temperature for 30 minutes. Next, 40µL of each bacterial aliquot were separately added to 120µL of CO2-independent media enriched with 4mM L-glutamine (no plasma), as positive controls with ~200 CFUs/50µL, which were also incubated for 30 min at room temperature. Finally, 50µL of all plasma-bacteria combinations and controls were plated onto tryptic soy agar plates (VRW International) in duplicate and spread using a sterilized spreading wand. Plates were incubated at 37°C for 16 hours, at which time plates were removed to count the number of CFUs per plate. The average of the 2 plates per sample, including the controls, was taken; the bactericidal capacity for each sample was then calculated as:

$$\frac{mean\ CFU\ control - mean\ CFU\ sample}{mean\ CFU\ control}$$

All samples were vortexed prior to and following the addition of a substance and prior to plating to ensure adequate mixing and to prevent bacterial clumping. Due to the ease of contamination in BKAs, proper precautions were taken to ensure sterility, including autoclaving of all materials prior to analysis, switching of pipette tips between every measurement, and sterilization of wands between each plate using a bead sterilizer (Electron Microscopy Sciences Germinator 500 110 volt, Hatfield, PA).

Innate Immunity—phagocytic activity

An *in vitro* carbon clearance assay was used to evaluate phagocytic activity of macrophages, a function of innate immunity, following Spinu et al. (1999) with slight modifications. Upon sample collection, 150 uL of each whole blood sample were added to individually-labeled tubes containing 3mL 0.09% saline for use as a background. The remainder (850 uL) of each sample (1:144 dilution) received 5.9 uL of India Ink (Speedball Art Products, Statesville, North Carolina) supernatant that had been centrifuged at 3000g for 30 min. Each of these samples were then vortexed to mix adequately then divided into 5 equal aliquots of approximately 170 uL each into 1.7 mL microcentrifuge tubes (Corning). Each of the five tubes from each bird were incubated for 10, 20, 30, 45, and 60 minutes at 37°C. After incubation, 150 uL of each blood and India Ink aliquot were added to pre-labeled (e.g. Bird: X, Incubation Time: X) glass tubes containing 3 mL 0.09% saline. Each of these samples were then centrifuged at 50 g for 4min. The absorbance of the supernatant of each sample was read with a spectrophotometer at 535 nm (SpectraMax Plus 384 multiplate reader, Molecular Devices, Sunnyvale, CA), using the background of each individual as a blank prior to reading the blood and India Ink supernatant. Optical density readings were converted to a log2 scale and phagocytic index was taken as the negative of the slope of the regression of optical density (log2) on time (min) for each bird.

Adaptive immunity—phytohemagglutinin (PHA) skin test

A PHA injection assay was used to evaluate T-cell-mediated immunity following modifications to Smits et al. (1999). After the injection of PHA, T-cell migration into tissue is part of a localized inflammatory response. At day 150, 38 days after the nestlings were assigned to their diet treatment groups, each bird was injected intradermally with 0.1mg PHA (Sigma Chemical Co., St. Louis, MO) that was suspended in 0.1mL phosphate buffered saline (PBS) into the toe web between the second and third digits. Toe web thickness was measured immediately prior and 24 hr post-injection using a pressure-sensitive micrometer and zeroing the micrometer between each measurement (Mitutoyo America Corporation, Aurora, IL); each toe web was measured twice, zeroing the micrometer between all measurements. All measurements and injections occurred on the toe web of the right foot, barring 2 individuals with open wounds on the plantar surface of their right feet for which we used the left foot to avoid confounding effects of ongoing inflammation. Toe web swelling was calculated as the difference in the average of the two measurements post-injection and pre-injection. To minimize experimental error, one investigator performed all injections and one performed all measurements. Smits et al. (1999) showed the unnecessity of a sham injection of PBS into the opposite toe web as a control, so this step was eliminated to minimize handling stress and the chances of an injection error.

Stress—plasma corticosterone analysis

Baseline plasma corticosterone levels were measured to evaluate baseline stress levels using an enzyme-linked immunosorbent assay (ELISA; Corticosterone ELISA Kit, Enzo Life Sciences) following Herring et al. (2011) with slight modifications. Kit instructions for small volume protocol for serum/plasma were followed, resulting in a 1:40 dilution of each plasma sample. Kit assay procedures were followed. The plate reader was blanked against the Blank Wells and optical density of each well was read at 405 nm (INSERT INFO ON SPECTROPHOTOMETER). Corticosterone Standard wells ranged from 32 pg/mL to 20,000 pg/mL; standards were used to calculate a standard curve on which sample concentrations of corticosterone were calculated using the mean optical density of each sample. All samples used in the analyses were collected within 3 minutes of capture to ensure an accurate representation of baseline levels (Romero & Reed 2005).

Stress—heat shock protein 70 (HSP70)

Concentrations of heat shock protein 70 (HSP70) in the blood were assessed as a secondary measure of baseline stress levels following Herring et al. (2011). Heat shock protein 70 concentrations were measured on day 165, 7 weeks after the nestlings were assigned to their diet treatment groups. Red blood cells were washed three times using phosphate buffered saline, centrifuged, and the supernatant was removed after the final wash. Red blood cell supernatant was then mixed with 1X extraction reagent and a protease inhibitor cocktail (Sigma, St. Louis, Missouri, USA), vortexed for 5 minutes, and then sonicated for 1 minute. Samples were again centrifuged (15 min, 2500 g) and the supernatant removed. We measured HSP70 in the supernatant using an ELISA kit (Enzo Life Sciences) specific to this stress protein. All samples were run in duplicate, and means of duplicates were used in all analyses.

Statistical Analysis

To improve normality (Shapiro-Wilk's test) and homogeneity of variance (Bartlett's test), we arcsine-square-root transformed all BKA and carbon clearance values. No other data required transformations. We used Welch's t-tests to compare pre- and post-diet treatment values for bactericidal capacity and corticosterone, as well as differences between diet groups for bactericidal capacity, corticosterone, HSP70, PHA, and carbon clearance, and differences in all measures by sex. We used linear regressions to assess differences by body condition (mass~tarsus length (MTL) residuals) and all immune and stress measures to each other. For each analysis, we used morphological measurements taken closest to each respective sample collection. We performed all analyses in R 3.4.1 Results are presented as means ± standard deviation (SD). Alpha=.05 for all analyses.

Results

Bactericidal Capacity

Bactericidal responses to *E. coli* (EBKA) were not significantly different between the anthropogenic diet group (0.61±0.53) and natural diet group (0.56±0.51) prior to the onset of the diet treatment or after the diet change at day 143 following admission to the PDRC. The change in EBKA from pre- to post-diet sampling did not differ significantly for either the anthropogenic ($t_{17.99}$=-0.707, p=0.49) or natural ($t_{17.99}$=-0.78, p=0.44) diet groups. Bactericidal response to *E. coli* was not influenced by mass, body condition, or sex at either sampling time point (Table 3.2).

Bactericidal responses to *Salmonella* (SBKA) also were not significantly different between the anthropogenic diet group and natural diet group prior to the onset of the diet treatment or after the diet change on day 143. Bactericidal responses to *Salmonella* did not differ significantly for either the anthropogenic ($t_{17.93}$=0.74, p=0.47) or natural ($t_{17.91}$=-1.02, p=0.32) diet groups from pre- to post-diet sampling. Neither mass, body condition, nor sex had an effect on SBKA at either sampling time point. BKA results are summarized in Table 3.2 and Figure 3.2.

Phytohemagglutinin (PHA) skin test

The PHA-induced toe swelling did not significantly differ between the two diet groups (anthropogenic: 0.91mm±0.44, natural: 1.03±0.40; $t_{17.80}$=-0.65, p=0.52) following the injection of PHA. Toe-web thickness was significantly thicker 24 hours following injection of PHA (1.93mm±0.46) than just prior to injection (0.95mm±0.15), demonstrating that swelling was successfully induced ($t_{22.85}$=-9.08, p=4.8e-9). Mass, body condition, nor sex had an effect on swelling (Table 3.2; Figure 3.4).

Carbon clearance

Phagocytic activity, represented by the calculated phagocytic index, did not significantly differ by diet group (anthropogenic: 0.061±.016, natural: 0.075±.03; $t_{13.61}$=-1.32, p=.21) when measured *in vitro* at day 149. Mass, body condition, and sex did not had an effect on phagocytic activity (Table 3.2; Figure 3.4).

Baseline corticosterone

Circulating corticosterone concentrations (ng/mL) were significantly different between the anthropogenic diet group (4.44±1.31) and natural diet group (2.96±1.15) at day 106, prior to the onset of the diet treatment ($t_{17.70}$=2.68, p=0.016). However, concentrations did not differ between the two groups at the post-diet sampling on day 143 (6.36±3.22 and 4.38±2.10, anthropogenic and natural diet groups respectively; $t_{15.35}$=-1.20, p=0.25) or for either the anthropogenic ($t_{17.93}$=0.74, p=0.47) or natural ($t_{17.91}$=-1.02, p=0.32) diet groups at pre- to post-diet change. The difference in the change in corticosterone from pre-diet to post-diet was not significant between diet groups (anthropogenic: 1.92±2.58, natural: 1.42±1.60; $t_{15.03}$=0.52, p=0.61). Baseline corticosterone was not affected by mass, body condition, nor sex. Summary of results in Table 3.2; Figure 3.3.

Heat Shock Protein 70 (HSP70)

Heat shock protein 70 levels (ng/mL) in birds maintained on the anthropogenic diet (1.59±0.15) did not significantly differ from those of birds maintained on the natural diet (1.73±0.27; $t_{13.77}$=-1.47, p=0.16). HSP70 levels were not influenced by sex, mass, body condition, or sampling order. There was no relationship between body temperature and HSP70 levels (adj-R^2=-0.049, $F_{1,18}$=0.113, p=0.74). A summary of these results is found in Table 3.2 and Figure 3.5.

Relationships between measures

There was a significant negative correlation between EBKA at day 143 and response to PHA (adj-R^2=0.16, $F_{1,18}$=4.72, p=0.043; Figure 4.6) and between SBKA at day 143 and response to PHA (adj-R^2=0.18, $F_{1,18}$=5.28, p=0.034; Figure 4.6). No other variables tested were significantly related to each other.

Discussion

In this study, we aimed to understand how an anthropogenic diet may affect several aspects of stress and immune function in a bird that has only recently become dependent on anthropogenic food resources, the American white ibis. Previous studies have shown that energy-rich diets improve overall health via a reduction in stress and an improvement in immunity (Wilcoxen et al. 2015; Strandin et al. 2018). Because anthropogenic ibis diets are typically less energy-dense, we expected an anthropogenic diet to have significant effects on immune responses and baseline stress, and birds subjected to an anthropogenic diet (via consumption of white bread) would show dampened immune function and increased indicators of stress. Contrary to these hypotheses, however, 30+ days on a simulated anthropogenic diet had no effect on any measure of immune function or stress. We also hypothesized there would be a negative relationship between baseline corticosterone and immune function, meaning that as baseline corticosterone levels increased, immune function would be dampened; however, this was not the case. We found no effect of any morphological or demographic parameter (body size, body condition, sex) on any measure of immune function or stress.

Bactericidal capacity in the nestlings was similar to levels recorded in wild juvenile ibis, measured by us in previous studies (Cummings (2018) and Curry (2017)). Similar to wild adult and juvenile ibis, the ibis nestlings had dampened bactericidal capacity against *S. paratyphi B* when compared to *E. coli.* While both have the potential to cause disease, *E. coli* is typically considered ubiquitous in avian environments and feces, whereas *S. paratyphi B* is not one of the top 20 serotypes most frequently isolated from wild ibis. Matson et al. (2006) propose that previous exposure to a pathogen can result in a heightened bactericidal capacity against that particular pathogen, which may explain the differences seen in the killing ability of ibis against *E. coli* versus *S. paratyphi B* —greater exposure to *E. coli* may result in greater bactericidal capacity against the bacterium (Matson et al. 2006). For these nestlings, we used similar plasma dilutions (1:15 for *E. coli*; 1:2 for *S. paratyphi B*) as were used in the field studies of wild ibis, indicating that nestling complement function is comparable to juvenile/adult ibis.

Our baseline plasma corticosterone levels were similar to those we found in adult wild ibis (Curry 2017; Cummings unpublished), providing evidence for the development of the HPA axis in white ibis. In some species, the HPA axis develops quickly and is responsive soon after hatching (i.e. black-legged kittiwakes) (Kitaysky et al. 1999). In other species, the development of the HPA axis develops is delayed, uncoupled, or, in general, slow (e.g. magellanic penguins) (Brian G. Walker et al. 2005). Our results suggest that the HPA axis in white ibis was developed some time before day 106, or our pre-diet sampling day, as we saw no differences in our pre- and post-diet baseline corticosterone values. These results are consistent with Herring et al. (2011), which found the HPA axis to be fully developed in free-living ibis nestlings relatively early compared to other altricial or semi-altricial species . Baseline corticosterone values were significantly higher in the anthropogenic diet group at the pre-diet sampling time point, when all birds were still being maintained on a natural diet. There were no husbandry changes, notable stressful events, or any other obvious reasons for this difference; both pens were maintained with identical cleaning, handling, and maintenance techniques, so this difference was likely caused by individual nestling differences. The post-diet sampling revealed no significant differences in baseline corticosterone between the two diet groups and the change in baseline corticosterone from pre- to post-diet was not different between the groups. We did notice, however, that the post-diet corticosterone levels spanned a larger range then the pre-diet values for both groups, but particularly the anthropogenic diet group, which may affect comparisons between and among the diet groups from pre- to post-diet change.

The lack of a clear relationship between diet within or among diet groups for any of the assays measuring stress or immune function may be explained by the length of time birds consumed the anthropogenic diet—it's possible that 30 days was not a sufficient period of time on the anthropogenic diet to detect physiological effects with our methods. Ibis in the wild experience fluctuations in resource availability due to fluctuating water levels, so this species may not be as sensitive to short-term changes in diet as one might expect. Captive diet manipulation experiments range in length, with some studies subjecting experimental animals to a certain diet for as little as 15 days (Acquarone et al. 2002) and some up to as many as 59 days or beyond after the start of experimental feeding (H. G. Smith et al. 2007). The latter of these studies involved manipulation of the protein content in the diet, and also found no effect of reduced protein on phytohemagglutinin-induced swelling in pheasants. However, pheasants fed higher protein diets displayed greater antibody responses, so it is also possible that the assays we used do not effectively capture the effects of an urban diet on white ibis physiology. Though our goal was to limit overall protein intake, we were providing a nutritionally balanced diet (20% seafood, 20% flamingo pellets, 20% eggs) in addition to the white bread (40%) which may have dampened the potential effects of the bread. If this is the case, it would have been beneficial to provide a lower proportion of nutritionally appropriate foods to limit the intake of specific micronutrients and further limit protein intake, which may have influenced assay results. However, maintaining nestling health was a priority in this experiment, and we wanted to prevent overt growth abnormalities. The nestlings exhibited preferences for the options available in their platters, and experimentally selected pellets last out of the available food options of bread, egg, and seafood, so further limitation of pellets may have been extraneous. It is known that while in urban habitats, ibis continue to forage on terrestrial invertebrates, so the effects of diet may be dependent on exact proportions of anthropogenic food to natural prey.

Our concurrent study with wild ibis in the field demonstrated that birds captured from urban habitats had increased bactericidal capacity against *E. coli*, as did birds consuming greater proportions of anthropogenic foods. We also found that adults captured from urban habitats had a dampened corticosterone response, and those consuming greater proportions of anthropogenic foods had lower baseline corticosterone levels in fall (Cummings unpublished). With these results in mind, the lack of effects seen in our nestling diet trials may also be explained by our inability to test any other urban-associated variables besides diet. Urban environments present social, environmental, and other changes for wildlife, and while diet is an important factor in these changes, it is perhaps the combination of them all that affect wildlife health and habituation to urban environments (Marzluff 2001).

When under stress, animals will release heat shock proteins which are primarily upregulated to protect cells from damage; higher levels of circulating heat shock proteins typically indicate higher baseline stress levels or a greater response to stress, both acute and chronic (Herring & Gawlik 2007). Heat shock proteins vary from corticosterone because it takes longer for HSPs to show up in the system and they remain circulating for longer periods of time; this makes them a useful measure for assessing long-term or chronic stress (Herring & Gawlik 2007; Martinez-Padilla et al. 2004). We saw no significant differences between HSP70 levels in the two diet groups, indicating similar baseline stress levels. While measuring heat shock protein concentrations provides a useful alternative to corticosterone, the amount of stress needed to induce an HSP response is often greater than what is needed to elicit an HPA response, so the amount of time on the anthropogenic diet or severity of the change in the diet may not have been adequate to induce a change in baseline HSP70 levels (Herring & Gawlik 2007).

We demonstrated a negative relationship between PHA-induced swelling and both SBKA and EBKA. Bacterial killings assays measure innate immunity while PHA measures adaptive immunity. Immune system components often undergo energetic tradeoffs, particularly between adaptive and innate immunity. Therefore, an enhanced innate response may result in a lack of energy allocated to the adaptive system leading to an attenuated adaptive response. Additionally, invasive species have been shown to have enhanced innate immunity in order to "expect the unexpected" in new environments (Lee & Klasing 2004). Ibis have recently (within the past 15 years) moved into urban habitats, so this may explain the enhanced innate response when compared to the adaptive response (Hernandez et al. 2016).

This experiment was used to assess the effects of an anthropogenic diet on stress and immunity in American white ibis nestlings. Resource availability and quality presents tradeoffs for urban wildlife and these results support the idea the immune components may tradeoff in response to resources. Additionally, this experiment provided insight into white ibis nestling development and reinforced the concept that urban habitats affect ibis in what is likely a combination of factors in which diet is just a small piece of the puzzle.

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Chapter 3 Tables and Figures

Table 3.1. A summary of the diet composition for the white ibis nestlings and the delivery methods used throughout the experimental design. "Days" is duration, with day 0 being their admittance to the PDRC (Athens, GA), for which nestlings consumed a specific diet phase. **Note:** all nestlings received the same diet (natural) until day 113, where the designated anthropogenic pen (n=10) began receiving white bread and reduced seafood content.

Diet phase	**N**	**Days**	**Delivery**	**Components**	**Protein (%)**	**Fat (%)**	**Carbs (%)**
Gruel (n=20)	20	0-14	oral gavage or hand fed	lake smelt, mazuri flamingo breeder pellets, dried egg yolk, CaCO3, Vionate, Stuart Thiamin E paste, Solgar chelated copper, water	38	13	0
Solid diet (n=20)	20	15-45	platters	lake smelt, mazuri flamingo breeder pellets, dried egg yolk, CaCO3, Vionate, Stuart Thiamin E paste, Solgar chelated copper, water	36	22	0
Solid diet + seafood (n=20)	20	46-113	platters	lake smelt, shrimp, whole crayfish, mazuri flamingo breeder pellets, fresh egg yolk, vionate	37	21	0
Final natural diet (n=10)	10	113-171	platters	20% lake smelt, 20% shrimp, 20% whole crayfish, 20% mazuri flamingo breeder pellets, 20% fresh egg yolk, <1% vionate	38	8	0
Final anthropogenic diet (n=10)	10	113-171	platters	6.6% lake smelt, 6.6% shrimp, 6.6% whole crayfish, 20% mazuri flamingo breeder pellets, 20% fresh egg yolk, <1% vionate, 40% white bread	20	16	36

Table 3.2.

Summary of the effects of diet, sex, mass, and body condition on both pre- and post-diet change measures of bactericidal capacity against *E. coli* (EBKA), bactericidal capacity against *S. paratyphi B* (SBKA) and baseline corticosterone (CORT) in white ibis nestlings exposed to either a simulated anthropogenic diet or a natural diet. Significant effects are in bold (α=.05). **Note**: means of groups are presented as mean±SD (for diet & sex).

Explanatory Variable	**Day 113 Measures (pre-diet)**			**Day 143 Measures (post-diet)**		
	EBKA	**SBKA**	**CORT**	**EBKA**	**SBKA**	**CORT**
Diet A: Anthropogenic (n=10) N: Natural (n=10)	A: .61±.53 N: .56±.51 $t_{17.90}$=.23 p=.821	A: .38±.25 N: .33±.16 $t_{15.41}$=.49 p=.634	**A: 4.44±1.31** **N: 2.96±1.15** **$t_{17.70}$=2.68** **p=.016**	A: .77±.52 N: .73±.50 $t_{17.97}$=.18 p=.859	A: .29±.24 N: .40±.15 $t_{15.35}$=-1.20 p=.25	A: 6.36±3.22 N: 4.38±2.10 $t_{15.48}$=1.623 p=.125
Sex M: Male F: Female	M: .51±.50 F: .67±.53 $t_{17.92}$=.629 p=.538	M: .32±.24 F: .38±.19 $t_{17.00}$=.632 p=.512	**M: 2.92±1.36** **F: 4.48±1.02** **$t_{16.69}$=2.89** **p=.01**	M: .70±.53 F: .81±.47 $t_{17.76}$=.523 p=.607	M: .32±.22 F: .38±.19 $t_{17.37}$=.710 p=.487	M: 4.39±2.23 F: 6.35±3.14 $t_{16.24}$=1.60 p=.128
Mass	Effect=-.0005 $F_{1,18}$=.261 p=.616	Effect=-.0003 $F_{1,18}$=.448 p=.512	Effect=.0002 $F_{1,18}$=.055 p=.816	Effect=-.001 $F_{1,18}$=2.078 p=.167	Effect=-.0004 $F_{1,18}$=2.07 p=.166	Effect=.0002 $F_{1,18}$=.055 p=.816
Body condition	Effect=<.002 $F_{1,18}$=.766 p=.393	Effect=<.002 $F_{1,18}$=.055 p=.816	Effect=.0002 $F_{1,18}$=.055 p=.816	Effect=-.0002 $F_{1,18}$=.0319 p=.8602	Effect=<.001 $F_{1,18}$=<.001 p=.99	Effect=.0002 $F_{1,18}$=.055 p=.816

Table 3.3. Summary of the effects of diet, sex, mass, and body condition on *in vitro* carbon clearance (CC), phytohemagglutinin skin test (PHA), and day 164 heat shock protein 70 concentrations in the blood (HSP70) of white ibis nestlings exposed to either a simulated anthropogenic diet or a natural diet. **Note:** means of groups are presented as mean±SD (for diet & sex).

Explanatory Variable	**Physiological Measure**		
	CC	**PHA**	**HSP70**
Diet A: Anthropogenic (n=10) N: Natural (n=10)	A: .061±.016 N: .075±.03 $t_{13.61}$=-1.32 p=.21	A: .91±.44 N: 1.03±.40 $t_{17.80}$=-.65 p=.52	A: 1.59±.15 N: 1.74±.27 $t_{13.77}$=-1.47 p=.16
Sex M: Male F: Female	M: .071±.026 F: .065±.023 $t_{17.74}$=-.52 p=.61	M: .96±.56 F: .97±.21 $t_{11.60}$=.073 p=.94	M: 1.59±.17 F: 1.73±.26 $t_{15.75}$=1.38 p=.19
Mass	Effect=1.81e-5 $F_{1,18}$=.27 p=.613	Effect=-.0009 $F_{1,18}$=2.27 p=.15	Effect=-.003 $F_{1,18}$=.76 p=.39
Body condition	Effect=.00014 $F_{1,18}$=2.13 p=.162	Effect=-.0003 $F_{1,18}$=.032 p=.86	Effect=-6e-5 $F_{1,18}$=.0094 p=.92

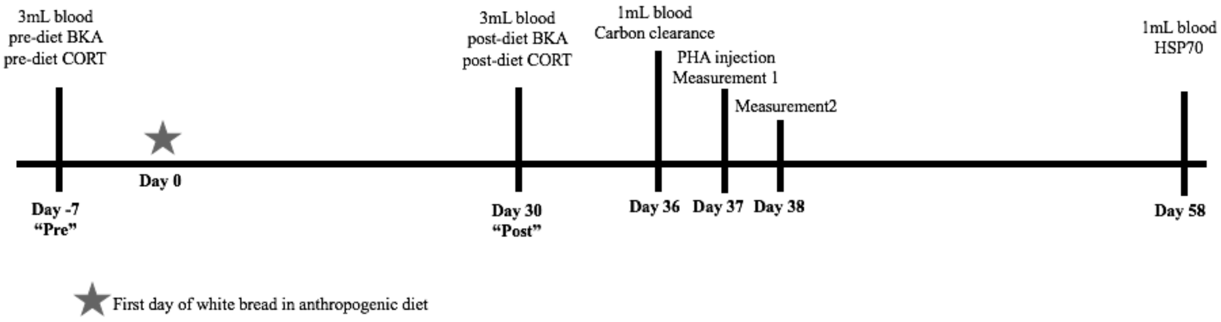

Figure 3.1. Timeline of white ibis nestling sample collection and the relevant assays samples were used for. Day 0 represents the day white bread was introduced into the diet and seafood content was significantly reduced for 10 of the nestlings**. Note:** distances between timeline items are not to scale.

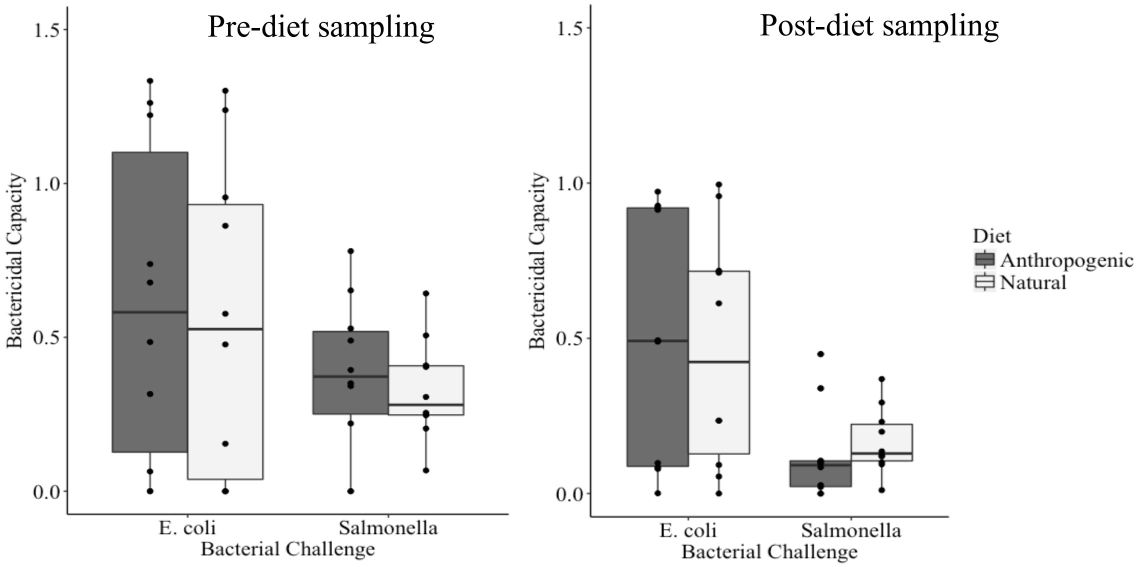

Figure 3.2. Differences in mean bactericidal capacity of white ibis nestlings (n=20) 7 days prior to the onset of an anthropogenic diet treatment for half of the nestlings (left) and 30 days after the anthropogenic group (n=10) had been on the new diet (right). There were no significant differences in bactericidal capacity between or among diet groups at either time point or with either bacterial challenge.

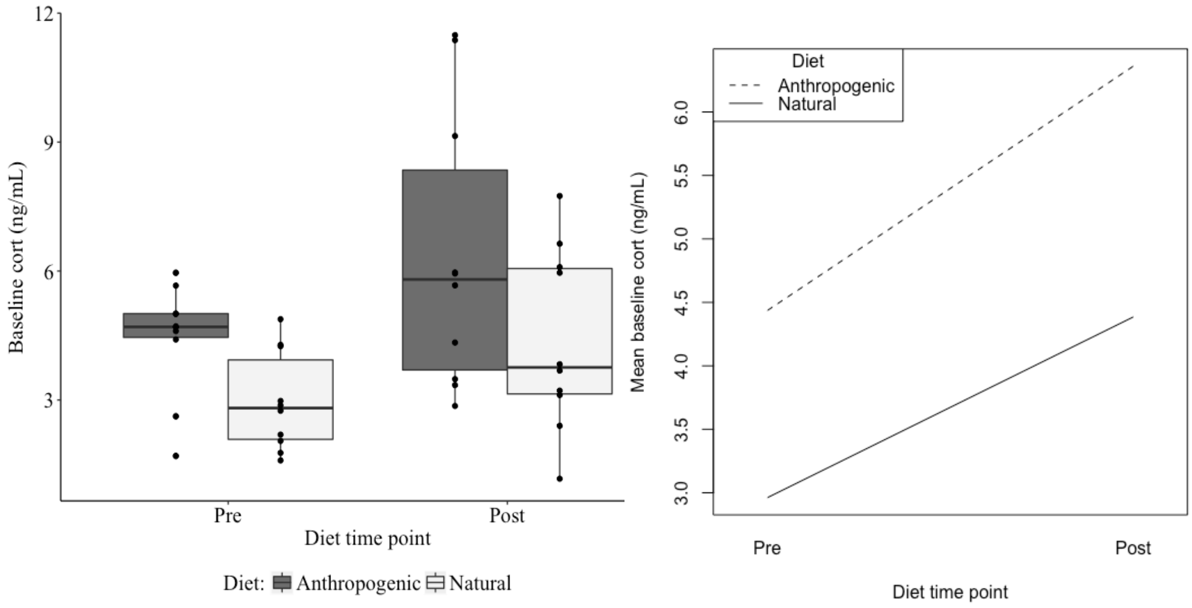

Figure 3.3. Left. Baseline corticosterone was significantly higher in white ibis nestlings (n=10) that would eventually be subjected to an anthropogenic diet than those that would be maintained on a natural diet (n=10) prior to the onset of that diet treatment for half (n=10) of the nestlings. However, by the post-diet sampling time point, 30 days following the diet change, baseline corticosterone was not significantly different between the groups. The post-diet sampling time point was not significantly different from the pre-diet sampling time point for either diet group. **Right**. The change in baseline corticosterone from pre- to post-diet for both diet groups. The difference from pre- to post-diet was not significant by diet group.

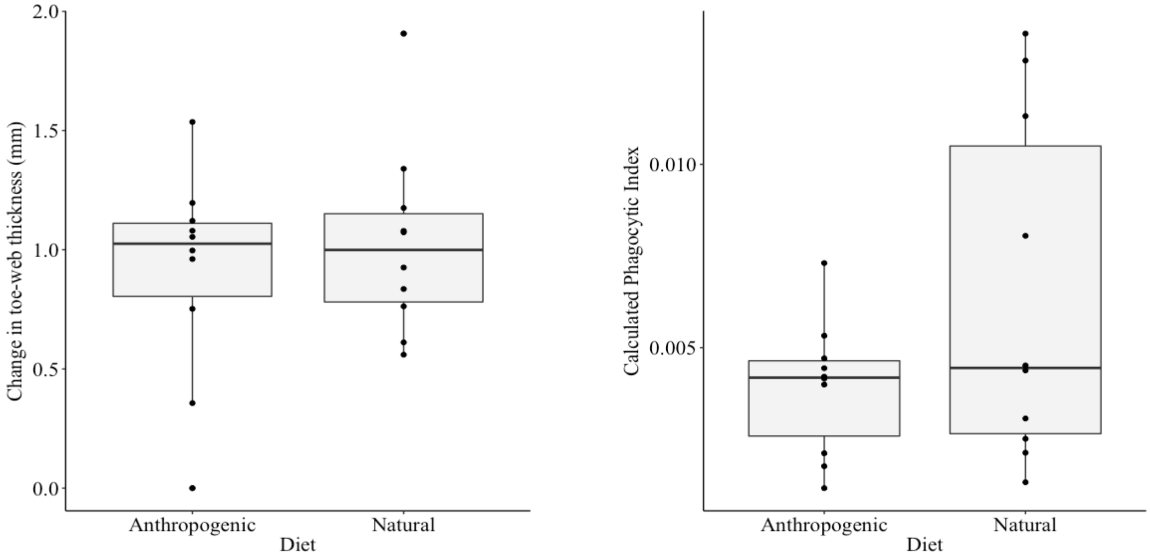

Figure 3.4. Left. There was no significant effect of an anthropogenic diet on PHA-induced swelling (as measured by the toe-web thickness prior to PHA injection subtracted from the toe-web thickness 24 hours following PHA injection into the toe web) in white ibis nestlings (n=20; 10 per diet group). **Right.** An anthropogenic diet had no effect on *in vitro* phagocytic activity as calculated by the negative slope of the regression of optical density of the supernatant at 5 time points (read at 535nm) in the nestlings.

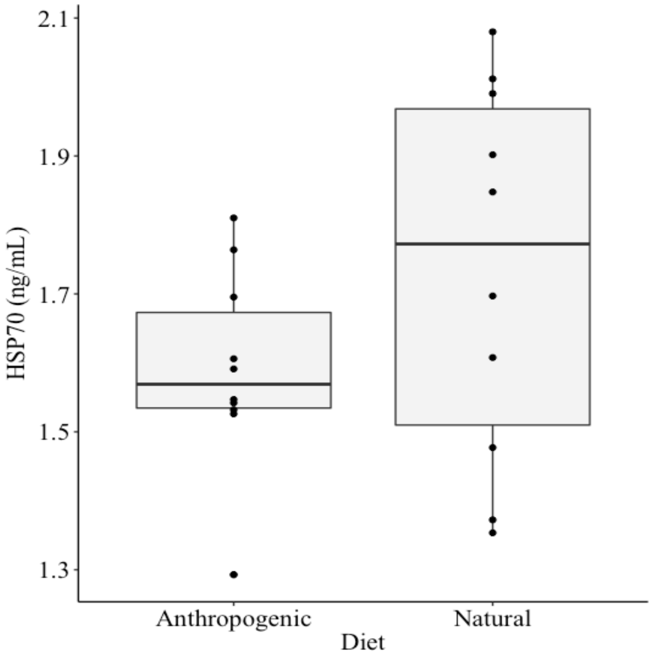

Figure 3.5. White ibis nestlings fed an anthropogenic diet had similar levels of HSP70 in their blood when compared to nestlings consuming a natural diet (n=20; 10 per diet group). However, HSP70 levels in nestlings on the anthropogenic diet spanned a wider range.

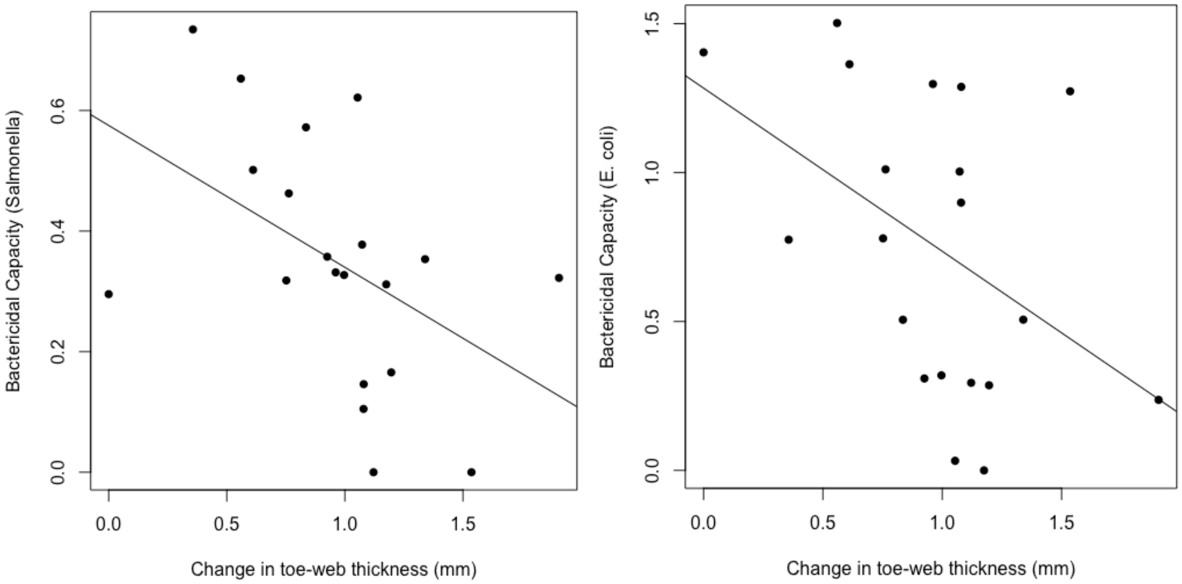

Figure 4.6. Left. There was a significant relationship between bactericidal capacity against *S. paratyphi B* and PHA-induced swelling in white ibis nestlings (n=20). **Right.** There was also a significant relationship between bactericidal capacity against *E. coli* and PHA-induced swelling.

CHAPTER 4

CONCLUSIONS

In this experiment, we sought to better understand how urbanization, with a particular focus on the resource provisioning, affects immunity and stress levels in a recently urbanized species, the American white ibis. Throughout south Florida, white ibis can be found foraging in a variety of urban habitats where they feed on items like white bread, chips, refuse, and other anthropogenic resources. We conducted a field experiment where we sampled ibis from a variety of urban and natural habitats and across a range of provisioning in the diet to determine how their immune function, baseline stress levels, and stress response varied. We found that birds captured from urban habitats had enhanced bactericidal capacity against E. coli, and adults captured from urban habitats had an attenuated stress response, indicating either habituation to urban-associated stressors or chronic stress causing a suppression of the stress response. We found that as resource provisioning in the diet increased, bactericidal capacity against E. coli increased, baseline corticosterone levels decreased in the post-breeding season, and the stress response increased in the breeding season. These changes indicate that urban environments and resources may be presenting potential tradeoffs for wildlife, possibly due to the benefits of reliable energy in the form of anthropogenic foods. To determine if the change in the quality of foods ibis obtain in urban habitats impacts is responsible for the observed physiological changes we saw in the field study, we exposed 10 ibis nestlings to an anthropogenic diet in captivity while 10 were maintained on a natural diet. We again measured immunity with BKAs as well as a phytohemagglutinin skin test, and in vitro carbon clearance and found no effect of diet on any of these measures. We also measured baseline stress levels via plasma corticosterone and heat shock protein levels in the blood and found no effect of diet on these either. These results indicate that the changes seen in the field are likely due to a combination of factors rather than due to diet quality alone. We also found that ibis immune components likely experience energetic tradeoffs and while innate immunity may be bolstered, it’s possible that the effects of urbanization and anthropogenic resource provisioning on other aspects of ibis immunity and health are masked due to these complicated tradeoffs.