THE UNIVERSITY OF CHICAGO

THE USE OF MULTIPLE BIOMARKERS TO UNDERSTAND THE EFFECTS OF URBANIZATION ON A FREE-LIVING PASSERINE, HOUSE SPARROW (*PASSER DOMESTICUS)*

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BY
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Dedicated to my beloved sister, Robin

Epitaph

"My mission in life is not merely to survive, but to thrive; and to do so with some passion, some compassion, some humor and some style." – Maya Angelou

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ACKNOWLEDGEMENTS

I dedicate this dissertation to my sister, Robin Carryl, who passed away in January 2007. I would like to believe that our family could have successfully emigrated to the United States without you being diagnosed with ovarian cancer, but that certainly is not the way life happened for us. Your illness created a pathway for mommy, daddy, Solita, Shawn, Shive and me to have access to what is commonly referred to as "The American Dream". This is truly for you, Robin. Your experiences were my source of strength to keep moving forward no matter the magnitude of adversity I faced. Losing you was the hardest thing I have ever experienced so any challenges thereafter simply paled in comparison. I did this for you and because of you. Forever grateful for your continued presence in my life.

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Chapter 1

General Introduction

Human-induced changes to the environment represent a new set of selective pressures on wildlife, with potential to affect their physiology (e.g., endocrine system, immune system, host microbiome), morphology (e.g., coloration, vocalization, body condition), and behavior (e.g., boldness, home-range size, nesting/denning choices). One large source of human-induced change is the rise in urbanization, which is defined as the conversion of natural habitats into areas partly covered by buildings, roads, sidewalks, and other impervious human constructions, that makes an area suitable for permanent and high-density human occupation United Nations (2018). The largely irreversible transformation of both habitat structure and ecological processes in urban areas is expected to act as a set of selective pressures, favoring organismal traits that are best suited to persist or even thrive near humans. These pressures mean that, to forestall local and perhaps permanent extinction, wildlife must adapt to these new conditions, such as through phenotypic plasticity or inter-generational microevolution.

For this dissertation, I sought to understand how varying degrees of urbanization impact the physiology of free-living avian species by focusing on three systems, namely the stress mediated HPA axis (the modulation of “stress hormones” such as corticosterone), the innate immune response (to a known pathogen, such as *E. coli*), and the gut microbiome (its diversity and community composition, and metabolic functions) [(see Chapter 2 for review)]. Importantly, I also wanted to test for direct and indirect links that might exist among these three systems, either amplifying adaptation or compensating for negative effects. In recent years, the assumed mutually reinforcing relationship among these systems has started to unravel: the consensus now is that they are connected, with no one of them truly operating independently to maintain homeostasis. Consider, for example, the modulatory effect that a host microbiome has on the immune system and the HPA axis via the promotion of maturation and maintenance of host immunity, and ultimately the microbiome's influence on an organism's ability to manage and respond to stress. An extensive study by Sudo et al. (2004) demonstrated the latter: higher levels of corticosterone were observed in germ-free (GF) mice when compared to specific pathogen-free (SPF) mice in response to a restraint stress test. The same researchers also showed a reduction in corticosterone levels among GF mice that were inoculated orally with fresh feces from the SPF mice. Similar results were shown for GF mice that received a bacterium called *Bifidobacterium infantis*. In another study, the administration of *Lactobacillus rhamnosus,* a bacterium known to induce the production of the neurotransmitter gamma-Aminobutyric acid, led to a reduction in circulating corticosterone levels and anxiety-like behaviors in mice Bravo et al. (2011). Conversely, some interactions between the three systems can be detrimental. Studies have shown that glucocorticoids can act as immunosuppressants through reducing cytokine synthesis and efficacy, which then results in the exaggeration of some infectious diseases (S. Cohen, Tyrrell, and Smith 1991; 1998; Leserman et al. 2000; M.L. Romero and Butler 2007). Glucocorticoids can also perturb the gut microbial community, which can reduce commensal bacteria that produce anti-microbial molecules responsible for limiting pathogenic noon and growth (D. Kelly and Conway 2005; Lei, Nair, and Alegre 2015; J.K. Evans et al. 2017).

Taken together, these results highlight the complex relationship that can exist among the stress mediated HPA axis, the immune response, and a host's microbiome. Although much of the knowledge on these interactions has been gathered from mammalian studies, some limited evidence suggests that a similar tripartite signaling network may exist in other taxa, including birds. The underlying basis for coregulating homeostasis, and therefore influencing wildlife adaptation and/or coping strategies, are the (1) shared biomediators (e.g., neurotransmitters, hormones), and (2) receptors that make the crosstalk among the stress mediated HPA axis, immune response, and host's gut microbiota possible. Microbes have been identified as producers and regulators of and responders to neurotransmitters (e.g., dopamine, serotonin, glucocorticoids, and catecholamines) that are also synthesized by neuroendocrine cells in hosts (Ortega et al. 2021).

To explore this possibility and to narrow the knowledge gap on physiologic responses to urbanization in general, this dissertation employs a mix of molecular analysis, isotopic analysis, standard physiological techniques, and experimental ecology to evaluate how a common wild bird, the house sparrow *Passer domesticus*, varies in these three aspects of physiology along an urbanized gradient. After a review of the issues of physiologic adaptation to urbanization (Chapter 2), I validate a methodological advance of feathers for multiple biomarkers (Chapter 3), I examine the relationship between corticosterone and diet (using the latter as a measure of urbanization) (Chapter 4). I then test the influence of stress-induced corticosterone on the innate immune response (Chapter 5), and finally consider technical pitfalls and potential solutions for studying free-living birds (Chapter 6).

When I first contemplated the interactions that might exist among these three physiological systems, it occurred to me that one system might compensate for the negative tradeoffs of one of the others if such a reaction was ultimately beneficial to the organism: that is, the net effects of all three might be most important, in contrast to a reductionistic approach. One possible scenario was the negative impact that elevated levels of corticosterone can have on body condition and how an individual might fine-tune other physiologies to cope. I speculated that this impact could be minimized if there was selection for gut microbes that, for example, were capable of extracting nutrients more efficiently, thus providing the animal with more energy, or were even capable of converting corticosterone to a less harmless metabolite. I also thought about the immunosuppressant role of corticosterone, which could be counterbalanced by a community of microbes capable of producing anti-microbial peptides to supplement host immune response. I visualized that a spectrum of different combinations existed having a positive net outcome for the individual, and, importantly, that these net-favorable combinations might in part explain the heterogeneity of corticosterone levels, immune response, and microbial community structure that have been reported in the urban ecology literature. For example, the presence of HPA-regulating microorganisms such as *Lactobacillus rhamnosus* and *Bifidobacterium infantis* might be a suitable alternative explanation for the common observation that corticosterone levels differ between urban and rural conspecifics (see Chapter 3).

These are the ideas and thoughts that I have contemplated and have continued to motivate my interest in interdisciplinary research. As I reflect, I now know there are many things I would have done differently but there lies my fatigue with hindsight – wishing what is known now could have been known and applied then. Nonetheless, I have accomplished my goals by advancing our understanding of: (1) the impact of varying degrees of urbanization on avian biological systems, and (2) how three important physiological systems work in concert to promote survival. My results: (a) show how multiple biomarkers (e.g., corticosterone and diet) can be quantified from a single homogenized sample (feathers), which places into ecological context; (b) demonstrate the ecological challenges and/or benefits assumed by an organism and likewise a population; and (c) challenge the "credit card hypothesis" that posits lower body condition and body mass among urban wildlife when compared to their rural counterparts. Furthermore, my work demonstrates that: (d) demonstrates that even within a single site, the effect of stress-induced corticosterone on the innate immune response is highly variable, and that (e) a dominant response may exist only when comparing among sites, that is testing across populations. Lastly, my work (f) highlights the tremendous challenge that remains in the extraction and sequencing of microbial DNA from avian as opposed to mammalian hosts, and thus the wealth of knowledge left to unpack in this growing field.

Chapter 2

Physiological responses of wild species to the Anthropocene: A review of human impacts on stress hormones, immune function, and the microbiome

2.1 The Anthropocene

The Anthropocene, the current phase of earth history, is characterized by human-induced change to the land surface, oceans, atmosphere, and life on Earth, and is evident globally (Crutzen 2002; Lewis and Maslin 2015). These human-induced environmental changes range from biogeochemical alterations, such as doubling the natural rate of terrestrial nitrogen fixation, to the evolution of life where extinction rates are 100 to 1000 times the natural background and are predicted to increase to 10,000x in the future (Crutzen 2002; Canfield, Glazer, and Falkowski 2010; De Vos et al. 2015; Teyssier 2020). The acceleration of extinction is referred to as the sixth mass extinction in Earth's history. There is also significant evidence supporting ongoing increases in temperatures, ocean acidification, novel precipitation patterns, habitat conversion (more than half of all land area has been altered by agriculture, forestry, grazing lands, and human settlement (Ellis and Revkin 2011), the overexploitation of species (particularly large-bodied and commercial taxa), land-degradation associated with mining and fuel production, seasonal hypoxia of waters linked to runoff of agricultural nutrients, pollution in general, and the intentional and unintentional introduction of species non-native ecosystems.

The ever-expanding effect of human activities on the globe is aligned with the exponential growth of human populations and especially with advances in agriculture and technology associated with the Industrial Revolution (1750-1850) and post-World War II (Steffen et al. 2011). The "Great Acceleration" describes the escalation of human impact since

1945 on Earth systems (e.g., carbon, nitrogen, and sulfur cycles) and the biosphere (McNeill 2016; McNeill and Engelke 2016). Human population size has experienced runaway population growth with less than 900 million people in the 1780, ~2 billion by 1930, and ~7.6 billion in 2020, with projected sizes of 8.6 billion in 2030, 9.8 billion in 2050, and 11.2 billion by 2100 (Nations 2018; McNeill 2016). Coupled with population growth and energy demands, human action since the mid-1700's has released 555 billion metric tons of carbon to the atmosphere, resulting in atmospheric CO_2 levels that have not been seen for at least 800,000 to several million years (Lewis and Maslin 2015). Likewise, nitrogen synthesized from converting atmospheric nitrogen to ammonia (e.g., the Haber-Bosch process) for use as fertilizers has risen from 4 million tons in 1950 to 85 million tons in 2015 (Canfield, Glazer, and Falkowski 2010; McNeill 2016). One major consequence of anthropogenic fertilization is the unused nitrogen (e.g., the nitrogen efficiency for wheat, rice, and maize crops is below 40%) that makes its way into marine ecosystems causing eutrophication, which leads to hypoxia in coastal waters (Diaz and Rosenberg 2008). In fact, the number of hypoxic systems ("dead zones") has doubled each decade since the 1960s and includes such major fishery areas as the Baltic, Kattegat, Black Sea, Gulf of Mexico, and East China Sea (Diaz and Rosenberg 2008). There has also been a >2000% increase in motor vehicles since the 40 million in 1950, and a 30,000% increase of plastic production between 1950 and 2015(McNeill 2016; McNeill and Engelke 2016).

Despite these negative impacts, the Anthropocene is set to continue, and human action will have ongoing impacts on Earth's ecosystems and biogeochemical cycles. A notable source of human impact includes the rise in urbanization, which is defined as the conversion of natural habitats into areas partly covered by buildings, roads, sidewalks, and other impervious cover that makes an area suitable for permanent and high-density human occupation (United Nations 2018).

Urbanization also transforms habitats for wild species, leading to greater pollution levels (e.g., light, chemicals, noise), reduced floral and faunal biodiversity (usually, although introduced species can compensate for loss of natives, or increase the total), higher (and different) anthropogenic food resources, and warmer temperatures (heat islands, with altered precipitation; (Arnfield 2003)) when compared to less densely populated peri-urban (or suburban), rural (largely agricultural), and still-wild (e.g., protected) areas. Although urbanization is recognized as a significant threat to wildlife, it is unlikely to halt or even slow -- as the human population continues to grow, more and more people will likely aggregate in cities, which have specialized infrastructures including for commerce. The United Nation’s 2018 report predicts that six out of every 10 people will be city dwellers by 2030, and that this number will rise to seven out of every 10 people by 2050.

The largely irreversible transformation of both habitat structure and ecological processes in urban areas is expected to act as a set of selective pressures, favoring organismal traits that are best suited to persist or even thrive near humans (Meillère et al. 2015; Alberti et al. 2017; Bailey 2021). These pressures mean that wildlife must either adapt (e.g., via changes to behavior, morphology, and physiology), such as through inter-generational microevolution or via phenotypic plasticity, or risk becoming extinct. The ability for organisms to adapt to urban habitats determines community composition there, with the subset of species unable to persist becoming urban avoiders while others become urban adapters and even urban exploiters (Blair 1996). Urbanization has led to reduced species richness within many groups including fish, invertebrates, and particularly in birds by selecting for such attributes as low natal dispersal, non-migratory behavior, reduced fear toward humans, and cavity-nesting (as opposed to ground-nesters), while selecting against diet specialist and low yearly fecundity (Paul and Meyer 2001;

Chace and Walsh 2006; Croci, Butet, and Clergeau 2008; Møller 2009; Callaghan et al. 2019). Among birds especially, urban conditions often select for generalist feeders rather than insectivores towing both to a reduction in native plant biodiversity and associated arthropod abundance, and to an increase in supplementary anthropogenic foods, e.g., backyard feeders (Threlfall et al. (2016); reviewed in Chace and Walsh (2006)).

Conditions in cities can also select species physiology. For example, interspecies comparisons among sister taxa have shown that the Bursa of Fabricius, which is a specialized immune organ in birds that reflects investment in immune response, is larger among urban birds than their rural counterparts (Møller 2009; Isaksson 2018). Møller (2009) posited that this difference in size of the Bursa of Fabricius allows urban bird populations to persist against more difficult conditions, such as higher rates of disease transmission associated with disproportionately high bird population density; that capability to persist might even have a positive feedback on population size. Consequently, North American bird biomass in urban areas is often largest among typically omnivorous species, both non-native species (e.g., rock dove, European starlings) and natives (e.g., American robins, Canada geese).

Urbanization can also drive evolution within a single species along an urban-rural gradient. For example, the “heat island effect” of cities resulted in a genetic increase in heat tolerance for the water flea *Daphnia magna* in urban freshwater ponds compared to rural ponds (Brans et al. 2017) and a similar adaptive thermal evolution was demonstrated for terrestrial acorn ants *Temnothorax curvispinosus* reared in urban areas (Diamond et al. 2017).

Among the tetrapods, birds are a primary favored focus for assessing and comprehending the effects of urbanization on wild animals and associated urban evolution. Like humans, birds are globally distributed, and many can migrate large distances, colonize novel areas, and occupy a large variety of ecological niches (Chung et al. 2018). Comprising ~10,828 species (Tietze 2018) and originating in the mid-Cretaceous about 100 million years ago (reviewed in Hird (2017)), birds today have highly variable morphologies (e.g., bright flamboyant colors, distinct beak shapes and sizes) linked to their diverse life history traits, and have economic (e.g., eco-tourism) and social (e.g., countries tend to have a national bird) significance. Furthermore, despite constituting only a minute proportion of the total living biomass on earth – ≈0.002 gigaton of carbon (Gt C) out of the ≈550 Gt C (Bar-On, Phillips, and Milo 2018) – the identifying, tracking, and observing of wild bird species is accessible to amateurs and professionals alike, resulting in one of the best-documented phylogenetics of all animal classes (Møller, Fiedler, and Berthold 2010) . For these reasons, birds are valuable sentinels for quantifying the impact of urbanization on wild species.

2.2 Endocrine Activity: Glucocorticoids as biomediators for adaptation

The endocrine system is a network of cells, tissues, and glands that produce and secrete the hormones that both regulate daily functions and mediate transitions between life-history stages (Adkins-Regan 2013). The role of stress hormones (i.e., catecholamines such as epinephrine and norepinephrine, and glucocorticoids such as cortisol and corticosterone) is particularly important because an organism's ability to survive is in large part a function of its capacity to respond to and cope with the hourly, daily, and seasonal demands of stress stimuli. The class of steroid hormones underlying the glucocorticoid response of animals is the most studied stress response in ecology, evolutionary biology, and conservation. Glucocorticoids are steroids that have a 4-ring carbon backbone with a variety of hydroxyl groups and carbon sidechains attached. They are present and functionally identical in all vertebrates and bind to the same guanine-protein-coupled receptors (here after G-receptors), but most species have only one as their primary glucocorticoid: corticosterone is primary in birds, reptiles, and amphibians, whereas cortisol is primary in fish and most mammals (M.L. Romero and Butler 2007).

The glucocorticoid response is characterized by a negative feedback loop (Fig. 2.1) referred to as the hypothalamic-pituitary-adrenal axis (HPA axis). The detection of a stressor activates the HPA axis in vertebrates to release corticotropin-releasing factor (CRF) and arginine vasopressin (AVP; referred to as arginine vasotocin in Aves and other non-mammalian classes) from the hypothalamus. Subsequently, CRF and AVP stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, and then ACTH directly induces the release of cortisol and corticosterone from the adrenal gland (M.L. Romero and Butler 2007). Cortisol and/or corticosterone can then regulate bodily functions (e.g., increasing blood glucose levels) and behaviors (e.g., fleeing or hiding) to prepare an individual to cope with the demands of the stressor. If the stressor ceases then cortisol and/or corticosterone inhibits further secretion of ACTH, thus restoring the HPA axis to homeostasis (Holt and Peery 2010; Sudo 2014). However, if a stressor persists, then glucocorticoids can lead to pathological effects that can jeopardize survival.

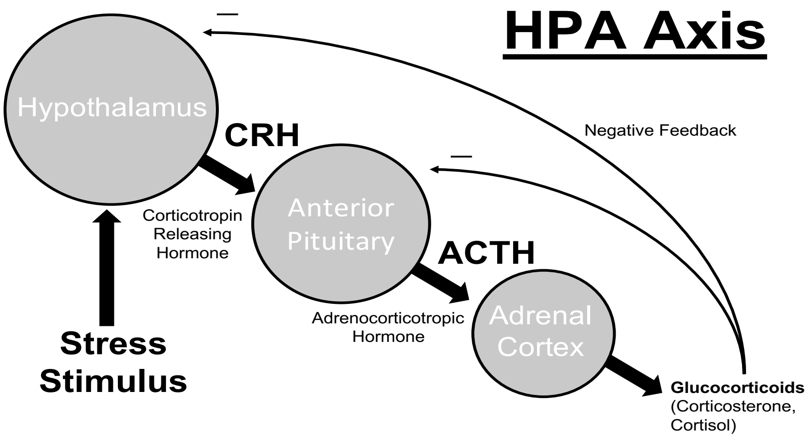


Figure 2.1 The negative feedback loop for the hypothalamus-pituitary-adrenal cortex axis.

Studies in birds have shown that corticosterone (the primary glucocorticoid in Aves) production changes frequently to meet a suite of daily to seasonal demands such as finding mates (Silverin 1998), fleeing, or fighting predators (Boonstra et al. 1998; Scheuerlein, Van't Hof, and Gwinner 2001), and investing in parental care (reviewed in Angelier and Chastel (2009)). In fact, an acute stress response – namely the short-term production of corticosterone with an efficient negative feedback – is considered adaptive because it promotes gluconeogenesis to meet the energy demands for reproduction and survival (Exton 1972; Dallman et al. 1993; Lattin and Romero 2015); however, see L.M. Romero and Beattie (2021). Acute stress is also believed to improve immune function in the short-term (Zylberberg 2015). However, a chronic stress response, i.e., the repeated or prolonged elevation of corticosterone, is often considered maladaptive because it can both inhibit secretion of growth and reproductive hormones and reduce their sensitivity in target cells, which result in lower body condition and decreased fitness, respectively. Moreover, as reviewed in M.L. Romero and Butler (2007), corticosterone also has broad and cascading inhibitory effects on the immune system. These effects include reducing the synthesis, release, and efficacy of cytokines and macrophages, which leads to the premature death of T and B cells by lowering the circulating levels of lymphocytes and reducing the number of phagocytic cells at inflammation sites. Given the combination of adaptive and maladaptive consequences of corticosterone release and elevation, researchers have hypothesized that wildlife individuals living under chronic urban environmental stressors (e.g., human presence, traffic noise) must achieve an attenuated stress response (e.g., reducing their responsiveness to stressors) coupled with an efficient negative feedback to persist under the novel challenges of urbanization (Bókony et al. 2021). This hypothesis that urbanizing pressures can select for the optimal phenotype is a key motivator for this dissertation. I seek to understand how differences in urbanization impact coping strategies via the individual, bidirectional, and tripartite relationship among corticosterone, the innate immune response and host microbiome.

When compared to less urban conspecifics, the most traditional prediction is that urban animals have (a) high baseline corticosterone levels due to chronic anthropogenic stressors continuously inducing a stress response, coupled with (b) a dampened elevation of corticosterone during an unpredicted stress event. It is believed that this resulting strategy helps organisms persist under highly urbanized areas by allowing them to minimize the negative effects of chronic stress (Bonier 2012; Foltz et al. 2015; Angelier et al. 2016; Bókony et al. 2021). This concept entails a baseline corticosterone level associated with daily (usually subconscious) energetic demands (e.g., arousal, foraging) that accumulate to reflect an individual's allostatic load. In other words, the greater the energetic demands, the greater the allostatic load on an organism that has experienced ongoing stressors. Thus, maintaining elevated corticosterone levels to cope with daily stressors increases “the wear and tear on the body” (Bonier 2012); see reactive scope model in L.M. Romero, Dickens, and Cyr (2009).

This baseline contrasts with stress-induced corticosterone levels that are linked to survival under unpredictable circumstances: the more sensitive or reactive an animal is to a pulsed stressor, the greater the magnitude of its stress-induced corticosterone. An excellent example is the association of stress-induced corticosterone levels with a greater fearfulness towards humans and reduced boldness (Atwell et al. 2012). Rural birds demonstrated a greater magnitude of corticosterone release associated with their greater fear of humans (e.g., flight-initiation distance was larger among rural than urban birds; in addition, urban birds demonstrated higher exploratory behavior scores associated with their lower induced corticosterone levels when placed in a novel environment).

These patterns are, however, not consistent and may reflect the range of variability organisms leverage to cope with stressors. For instance, when comparing urban and rural populations, some studies found no difference in stress-induced corticosterone levels (e.g., Atwell et al. (2012); Meillère et al. (2015)) while others found lower, higher, or no difference in baseline corticosterone (Schoech et al. 2007; Atwell et al. 2012). Such inconsistencies suggest that current predictions of corticosterone levels along urbanized gradients are too simplistic or deterministic. To evaluate this variability in study outcomes, Injaian et al. (2020) used HormoneBase (Vitousek et al. 2018) to conduct a large-scale comparative analysis, testing for a general relationship between corticosterone levels (both baseline and induced) and anthropogenic challenges. They found a significant but very weak negative relationship between anthropogenic noise levels and baseline corticosterone (n = 344 measures from 61 avian species; b-estimate = -0.36; 95% CI = -0.68, -0.03 for urban avoiders * noise and b-estimate = -0.13; 95% CI = -0.83, 0.56 for urban exploiters * noise). Furthermore, the results from a MCMC glmm showed no support for a general association, either positive or negative, between stress-induced corticosterone levels and human-induced environmental changes. The researchers thus cautioned against directional predictions: high or low corticosterone levels are not necessarily associated with high or low exposure to urban challenges. It may be more reliable to look beyond the traditional expectation of a linear relationship between corticosterone levels and urbanization. Another strategy to consider, which was implemented into this dissertation research, is integrating multiple physiologic variables to comprehend how wild, free-living birds are coping with urban stressors (L.M. Romero and Beattie 2021).

2.3 The immune system as a measure of adaptation

The avian immune system has evolved to protect against and fight pathogenic microorganisms while concurrently selecting a microbial gut flora that can boost the host's immune response and health (Genovese et al. 2013; Al-Khalaifah and Al-Nasser 2018). This immune ability is partially governed by the concept of "discrimination between self from non-self" that was first proposed by virologist Sir Frank Macfarlane Burnet in the 1940s (but see Pradeu and Carosella (2006); self/non-self is reviewed in Cooper (2018) and Gonzalez et al. (2011)). The avian immune system, like that of others in the vertebrate lineage, has evolved an innate and adaptive mechanistic arm that works synergistically to mount an effective immune response (E.L. Cooper 2010; Buchmann 2014; E.L. Cooper 2018). Because of shared ancestry and series of selective pressures, many commonalities and differences also exist among the immune systems of birds, mammals, fish, and amphibians (reviewed in Boehm and Swann (2014); Kasahara and Sutoh (2014)).

Referred to as "fast acting ancient molecules" by Buchmann (2014), the components of the innate immune response are the first line of non-specific defense (including physical defense) against pathogens. These components are made up of molecules that likely evolved in unicellular organisms as much as 1 billion years ago (Buchmann 2014; E.L. Cooper 2018)). In fact, it is believed that for the first unicellular organisms to prevail, they had to have evolved this "self from non-self" detection strategy. Take for instance, the ability of an organism to identify what is food, what is a toxin, and what is self, based on cellular receptors that can bind to foreign materials and provide feedback (Buchmann 2014). Once self and non-self are distinguished, the process of phagocytosis allowed these unicellular organisms to digest materials for energy or expel materials that threatened survival.

The molecules in the innate immune system include humoral properties (e.g., lysozyme, natural antibodies, and antimicrobial peptides – defensins, cathelicidins) and cellular properties [e.g., lymphocytes, macrocytes, and phagocytes – neutrophils (heterophils in birds), monocytes, macrophages] whose primary functions are to detect, isolate, and eliminate microorganisms that pose a threat of infection and disease (Aristizábal and González 2013; Riera Romo, Pérez-Martínez, and Castillo Ferrer 2016). Not surprisingly, there are homologs of innate immune system properties found in plants, invertebrates (e.g., insects, mollusks, worms, and spiders), and primitive vertebrate ancestors (e.g., hagfish and lampreys).

In the event that the innate immune system is unable to eliminate the microbial threat and the pathogen persists, the adaptive immune system (also referred to as the acquired response) is activated through a cascade of cellular interactions. The adaptive immune system is exclusive to vertebrates and so likely evolved far more recently than the innate immune response (Fig. 2.2). It is estimated to have arisen ~450 million years ago in jawed vertebrates (Flajnik and Kasahara 2010; Buchmann 2014; N.C. Smith, Rise, and Christian 2019); but see the unique variable lymphocyte receptors in jawless vertebrates such as hagfish and lamprey discussed in Sutoh and Kasahara (2020). The adaptive immune response may take hours or even days to create its pathogen-specific response in contrast to the non-specific innate immune response that can occur within minutes (Helin 2020). However, the sophistication of the adaptive immune response lies in its “memory” (also referred to as anticipatory immunity; M.D. Cooper and Alder (2006)). Once initiated, the adaptive immune system creates a unique antigen receptor for microbicide and stores this information for subsequent rapid deployment in case of future re-encounters with the same pathogen – this memory is the basis of vaccination (Buchmann 2018). The properties that carry out this effective response are lymphocytes (e.g., T-cells and B-cells), immunoglobulins, and the major histocompatibility complex molecules (Kaiser and Balic 2015).

Along with these specialized cellular and humoral immune components are the specialized organs that produce and/or store them. Of particular interest are the similarities between the immune organs of birds and mammals, despite their having diverged from their last common ancestor more than 200 million years ago (Brusatte, O'Connor, and Jarvis 2015). For example, both taxa have a spleen and thymus, which produce antibodies and T-cells, respectively (Scanes 2020). Likewise, some unique features are exclusive to birds, such as the Bursa of Fabricius, which is the primary organ for producing B cells (Scanes 2020).

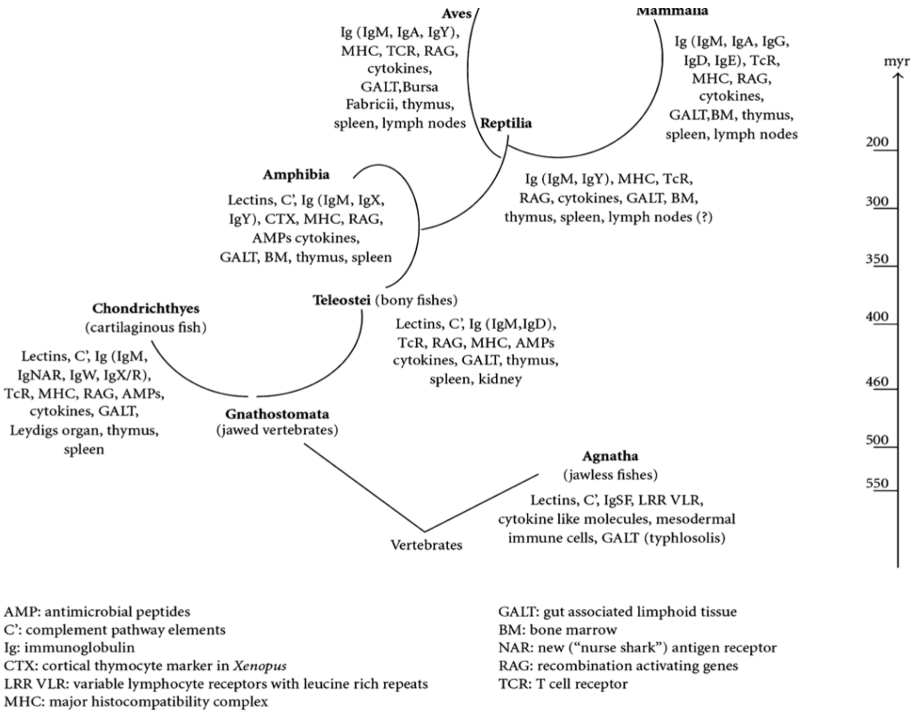


Figure 2.2 Evolutionary history of the innate and adaptive immune response across vertebrates. Reprinted from (E.L. Cooper 2010) under license CC BY-NC-SA 4.0. License number: 5083751234173

An immune response requires energy and thus organisms must allocate energy and resources in a manner that does not deplete their overall health. Analogous to other systems, a tradeoff exists between mounting and maintaining an immune response and other physiological demands. Consequently, immunocompetence is predicted to be affected by the degree of urbanization, given that this entails differences in chemical pollution, resource acquisition and nutrient value, and temperature, among other environmental conditions (reviewed in Martin et al. (2010)). Bobby Fokidis, Greiner, and Deviche (2008) demonstrated this effect in the curve-billed thrasher, *Toxostoma curvirostre,* and northern mockingbird, *Mimus polyglottos*: in urban settings, both species had higher heterophil to lymphocyte ratio (H/L) and higher leukocyte count than their rural counterparts, which suggested chronic stress and infection. Similarly, Cummings et al. (2020) showed that birds on an anthropogenic diet had significantly lower innate immune response to killing *Salmonella paratyphi* than those on a more natural diet. Conversely, Carbó-Ramírez and Zuria (2017) found no difference in H/L ratio between sites of varying degrees of urbanization and more over found that the less urban site had 2x the basophils concentration of the more urban site contrary to the expectations for pathogen loads.

2.4 Host microbiome and its role in adaptation

The complex microbial community that lives in and on hosts constitutes an important component of an animal’s ecology and evolution (Zilber-Rosenberg and Rosenberg 2008). Multiple diverse communities, especially of microbes (e.g., bacteria, archaea, metazoans, fungi, protozoa; viruses, helminth parasites), have built relationships with animals, living on their skin, scales, and feathers as well as within their respiratory, reproductive, and digestive tracts (Do Yup Lee and Choi 2015). Methodologically, the transition from culturing to high-throughput genetic sequencing has transformed our understanding of ‘microbiomes’, allowing for a comprehensive analysis of an organism’s microbial community structure (relative abundances of taxa), composition (identity of taxa), and potential functions (Klomp et al. 2008; Do Yup Lee and Choi 2015). The classic culture-based technique probably biased against ~99% of microbial species due to growth media limitations (Amann, Ludwig, and Schleifer 1995; Rinke et al. 2013). In contrast, recent culture-independent techniques utilize marker gene amplicons (e.g., the highly conserved 16S rRNA gene in bacteria) to generate profiles of the relative abundances of microbes, and ‘shotgun’ metagenome sequencing used to infer microbial gene functions (Kohl et al. 2013) have revealed complex and fascinating host-microbe relationships. Thus, once viewed exclusively as disease-causing agents, microorganisms are now appreciated as having a wide range of symbiotic – and thus positive -- relationships with their hosts, which include the regulation of a host’s immune maturation (Kim 2018), the defense against pathogens (Tsolis and Bäumler 2020), host metabolism such as nutrient uptake and storage (Krajmalnik-Brown et al. 2012), the degradation of toxins (Shukla and Beran 2020), aspects of animal behavior (Ezenwa et al. 2012), and the endocrine system (Neuman et al. 2015; Williams et al. 2020).

Animal life is inextricably linked to its ongoing relationship with microorganisms, and there is growing empirical evidence that the relationship can increase fitness, including among birds. For example, in hoopoes *Upupa epops*, secretion of an antibody-rich solution from the uropygial gland has been associated with hatching success arising from reduced growth of microbial pathogens on the surface of eggshells (Soler et al. 2008; Martín-Vivaldi et al. 2009; Magdalena Ruiz-Rodríguez et al. 2014). Researchers discovered that the oily secretion that coated the eggs was rich in an antibiotic-producing bacterium known as Enterococci (M Ruiz-Rodríguez et al. 2009; Magdalena Ruiz-Rodríguez et al. 2012).

One of the best described host-microbiome relationships is that of the microbiota gut-brain-axis, a bidirectional interaction between the host brain and gut microbiota (Fig. 2.3). Gut-associated lymphoid tissue (GALT) in the gastrointestinal tract is one of the substrates for the connection between immune function (70-80% of the body's immune cells is in the GALT), gut bacteria and stress related HPA molecules (de Weerth 2017). As reviewed in Foster, Rinaman, and Cryan (2017), integration of the microbiota gut-brain axis, relies on afferent and efferent neural projection pathways, biological mediators (e.g., neurotransmitters), immune signaling molecules (e.g., antibodies), altered intestinal permeability, modulation of enteric sensory-motor reflexes, and entero-endocrine signaling. Studied most profoundly in mammals through experimental immune and stress challenges as well as observational and germ-free models, the gut-brain-axis is believed to be an important regulator of a host's stress responsiveness (Sudo et al. 2004), pain modulation (Chichlowski and Rudolph 2015), and learning and memory ability (Davidson et al. 2018), and is also thought to be involved in the pathogenesis of diseases, such as irritable bowel syndrome (Mayer, Savidge, and Shulman 2014) and, perhaps, autism spectrum disorder (Pulikkan, Mazumder, and Grace 2019).

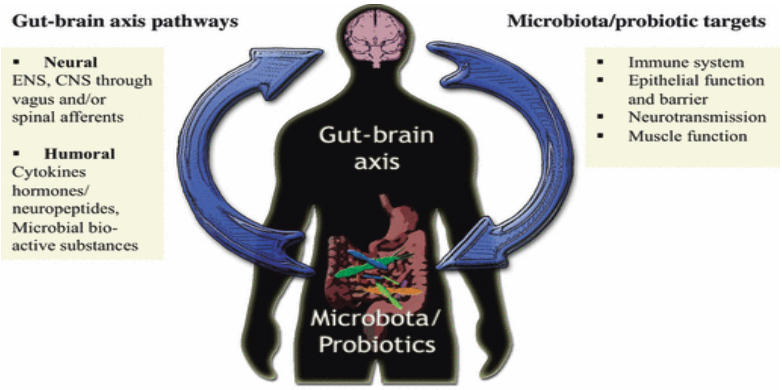


Figure 2.3 The proposed microbiota-gut-brain axis pathway. Reprinted from (Bercik, Collins, and Verdu 2012) under license CC BY-NC-SA 4.0. License number: 5084380912322.

One important study showed that severing the connection between the central nervous system and the gut (i.e., cutting the vagus nerve) resulted in a decreased risk for Parkinson's disease (Svensson et al. (2015); reviewed in Liu and Forsythe (2021)).

It is speculated that the microbiota-gut-brain axis documented in mammals also exists in birds, although its degree of similarity is still unknown. Slevin et al. (2020) published some of the earliest evidence that supported (although admittedly not very strongly) an avian microbiota-gut-brain axis, finding that bacterial genera differed in abundance among zebra finches *Taeniopygia guttata* having different cognitive ability. They found that poor-performance males had greater proportional abundance of *Helicobacter* (44.6%) than medium- and high-performance males (29.3% and 10.6%, respectively), and that poor-performance females had higher *Gallibacterium* abundance (3.1%) than medium- (1.1%) and high-performance females (0.02%); both microbial genera are mostly pathogens. Thus, although *Helicobacter* and *Gallibacterium* may indicate host quality, it is possible that they may also directly or indirectly influence avian cognitive ability.

The interaction between hosts and their associated gut microbiota has brought new insights to how urbanization affects free-living birds, especially since it is well-documented that the microbiome is shaped in part by the environment (Lucas and Heeb 2005; Benson et al. 2010; Archie and Theis 2011; Magdalena Ruiz-Rodríguez et al. 2014). Studies have demonstrated that urbanization significantly alters the gut microbiome of birds living along an urbanized gradient (Teyssier et al. 2018; Phillips, Berlow, and Derryberry 2018; Gadau et al. 2019; Knutie, Chaves, and Gotanda 2019; Teyssier et al. 2020; Berlow, Phillips, and Derryberry 2021). For instance, Teyssier et al. (2020) found that house sparrows, *Passer domesticus,* in urban settings had a lower gut microbial species diversity and a microbial composition that was associated with highly processed and low-fiber foods often found in cities. This finding corroborated the results of the first study to investigate the effects of urbanization on the microbiome of a wild avian host (Teyssier et al. 2018), as well as of a similar study on two species of Darwin's finches (*Geospiza fuliginosa* & *G. fortis*) in the Galapagos (Knutie, Chaves, and Gotanda 2019).

Increased microbial richness often implies better body quality and/or better health conditions because it may be associated with wider access to essential metabolic functions that can, for example, increase nutrient uptake and support immune functions (Le Chatelier et al. 2013). High microbial diversity may also condition hosts to be more resilient against pathogenic growth (Litvak and Bäumler 2019), although this correlation does not always hold. For example, white throated sparrows *Zonotrichia albicollis* have been found to have higher microbial richness in urban than in rural individuals that is unrelated to body condition (Berlow, Phillips, and Derryberry 2021); moreover, an analysis of Darwin's finches did not find a positive correlation between body mass and bacterial diversity (Knutie, Chaves, and Gotanda 2019). Beyond richness, some evidence exists that urbanization affects the taxonomic composition and metabolic functions of avian microbiomes. Teyssier et al. (2018) found a positive correlation between the metabolic function (blasted against KEGG database) of xenobiotic biodegradation and urbanization in house sparrows *Passer domesticus,* suggesting an increased capacity to deal with the environmental toxins and chemical pollutants that are most associated with urban environments. Taken together, these studies indicate the potential for species-specific microbiome responses to urbanization, that vary with multiple extrinsic factors (such as diet and differing landscapes) interacting with a range of intrinsic factors such as the immune and endocrine system.

2.5 The interplay among stress-immune-microbiome

Studies have shown that as individual systems, the stress mediated HPA axis, immune response, and host's gut microbiota are critical for promoting and maintaining homeostasis as well as exacerbating pathologies. However, there is compelling evidence that a complex tripartite signaling network exists among them that may be rooted in shared evolutionary ancestry (Sharpton 2018; Ortega et al. 2021). Among the evidence to suggest coregulation, and therefore influence on wildlife adaptation and/or coping strategies, are the (1) shared biomediators (e.g., neurotransmitters, hormones), and (2) receptors that make the crosstalk among the stress mediated HPA axis, immune system, and host's gut microbiota possible (Fig. 2.4). It is therefore my prediction that selective pressure to optimize the integration of stress mediated HPA activity, innate immune response, and avian gut microbiota may exist to neutralize and counteract adverse effects of urbanization. Additionally, results may also indicate the tradeoffs among the stress-immune-microbiota network that can occur due to finite resources.

The full extent of the stress-immune-microbiota intercellular signaling mechanism is not yet known, but researchers have identified a few among them that can contribute to regulating homeostasis (Fig. 2.4). Noteworthy are the G-protein-coupled receptors that convey a stress mediated HPA response are also expressed by immune cells and microorganisms. Glucocorticoids, therefore, can bind to G-receptors on immune cells and microorganisms to modulate stress response or even stimulate the production/release of cytokines (Lyte 2010; Ortega et al. 2021). Furthermore, resulting cytokines can then stimulate the HPA axis including the release of ACTH from the pituitary gland (reviewed in Ortega et al. (2021)).

Likewise, immunoglobulin A, an antibody abundant in the gut mucosal surface, can select for commensal bacteria such as those that release metabolic products (e.g., short-chain fatty acids: butyrate, acetate, and propionate) (Bunker and Bendelac 2018; Silva, Bernardi, and Frozza 2020), which in turn go on to regulate other immunoendocrine functions (e.g., inflammation and stress HPA axis) (van de Wouw et al. 2018; Silva, Bernardi, and Frozza 2020). All in all, the influence one system can exert on the others via shared biomediators and receptors is profound. Thus, integration among these three systems drastically increases the number of

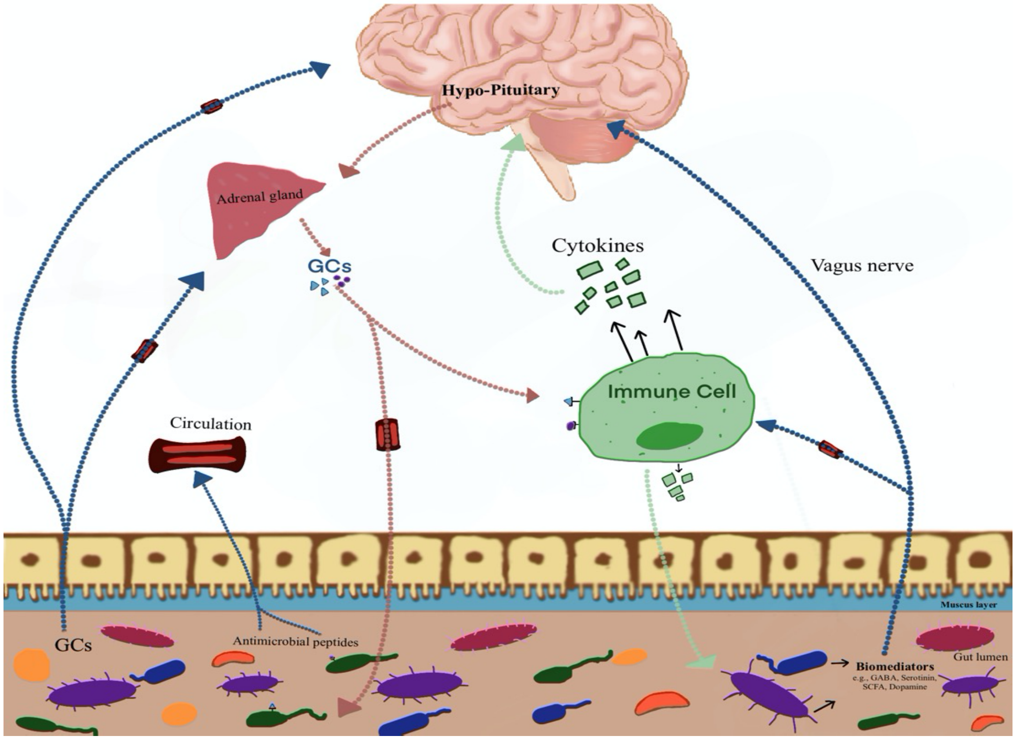


Figure 2.4 A hypothesized summary of the stress-immune-microbiota intercellular signaling mechanism that is made possible by the shared biomediators (e.g., glucocorticoids (GC), gamma-Aminobutyric acid (GABA), cytokines) and receptors (g-protein receptors). Circulating GCs produced by the stress HPA axis can bind to immune cells and bacteria, which may stimulate the production of cytokines. Likewise, gut bacteria may release biomediators (e.g., GABA) that can travel via the vagus nerve to modulate GC production from the HPA axis. The co-modulatory ability thus allows for an integrated response to challenges in an organism's environment for the effort of maintaining homeostasis. Illustrated by Sophia S. Carryl.

coping strategies organisms can employ to respond to environmental perturbations such as urbanization (Ortega et al. 2021).

Studies geared towards understanding the effects of urban pressures on wild animals must continue as urbanization is set to increase and is on the trajectory to become the most dominate landscape. Empirical evidence supporting the stress-immune-microbiota tripartite signaling network in wildlife animals can move the science of urban ecology beyond its traditional predictions towards examining the interactions that exists among these biological systems governing survival outcomes. There is no doubt that this quest is difficult, but with a concerted effort, researchers can illuminate the potentials and limitations of this tripartite signaling network, which can ultimately help conservation biologists predict and manage wildlife population declines.

Chapter 3

One sample two biomarkers: common glucocorticoid extraction methodology used in feathers does not affect the measurement or reliability of stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$)

3.1 Abstract

Human-driven changes to the environment are likely to continue having effects on wildlife ecology and evolution. Scientists must study the ramifications of these anthropogenic changes to determine the stability of ecosystems, which may require (early) interventions. Corticosterone and stable isotopes (e.g., $\delta^{13}C$ and $\delta^{15}N$) are two biomarkers used to monitor the health status of individuals and populations. Corticosterone reflects how wildlife species are responding to environmental perturbations, and stable isotopes indicate the dietary choices in their ecosystem. Together, measuring corticosterone and diet can provide information on the potential relationship between stress physiology and dietary resources within the same individuals. Currently, collecting sufficient samples (e.g., blood, feces) to measure both biomarkers is a limiting factor. However, using a single homogenous pool of feathers is a non-invasive alternative that may maximize the number of measurable biomarkers. Here, we tested whether the methanol-based corticosterone extraction process affects stable isotope values found in feathers from house sparrow (*Passer domesticus).* Our results showed that $\delta^{13}C$ and $\delta^{15}N$ remained unchanged by methanol based $CORT_f$ extraction. We then validated these results by demonstrating the dietary shift from juveniles' insect-rich diet to adults' grain-rich diet, wherein juveniles had significantly higher $\delta^{15}N$ and lower $\delta^{13}C$ values than adults. These results support a reliable and efficient methodology to provide complementary data on avian response to

environmental conditions, particularly in field ecology where samples may be scarce. Collecting multiple quantitative biomarkers can advance data-driven pathways to further understand changes to organismal traits in an increasingly urbanized world.

3.2 Introduction

A growing number of researchers have capitalized measuring corticosterone (herein, CORT) levels and stable isotope ratios in wildlife by using keratinous tissues such as feather and hair (Bearhop et al. 2002; Lattin et al. 2011; Fairhurst, Marchant, et al. 2013; Fairhurst, Vögeli, et al. 2013). CORT and stable isotopes (e.g., $\delta^{13}C$ and $\delta^{15}N$) represent two important biomarkers that can provide information on the health status of individuals and populations. CORT is often used to measure the dysfunction/stability of the hypothalamic-pituitary-adrenal axis (HPA axis) and can provide information on how individuals are responding to environmental stressors. Although the short-term elevation of CORT is considered adaptive, it is the long-term elevation of CORT that may have detrimental consequences on body condition, reproduction, and growth (Exton 1972; Dallman et al. 1993; Lattin and Romero 2015). Stable isotope ratios can provide isotopic signatures to estimate diet choices. Stable isotopes carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$), which are denoted as $\delta^{13}C$ and $\delta^{15}N$, respectively, have been applied to assess the dietary information of wild birds and mammals since the late 1970s (reviewed in Kelly (2000)). In urban ecology, carbon stable isotopes ratios are currently being used to determine whether wildlife animals are consuming more anthropogenic foods or natural foods (Newsome et al. 2010; Penick, Savage, and Dunn 2015). δ15N is useful to infer trophic levels and can relay how much protein is in an individual's diet. Together, CORT and stable isotope measurements can help census population health over time and can help to determine phenotypic differences among populations (e.g., urban versus rural populations) that can be used to understand adaptation and contemporary evolutionary processes.

Traditionally, ecological studies on wildlife rely on blood to determine CORT levels and observe feeding animals or analyze their gut contents to determine diet (Gavett and Wakeley 1986b; L.M. Romero and Reed 2005). However, both sample types represent a “snapshot” of an organism’s physiology, which cannot be easily extrapolated beyond the time of collection. It is also recognized that CORT in the blood could be biased by an investigator-induced stress response because of capturing and handling (L.M. Romero and Reed 2005; Bortolotti et al. 2008; Bortolotti et al. 2009; Lattin et al. 2011). Collecting blood and gut contents, while notably invasive techniques, also require special storage and quick access to below zero refrigerators. These biological samples may also be limited in quantity and so will most likely be used to measure a single biomarker. Conversely, during feather and hair growth, which may take days to months, circulating CORT and stable isotopes are incidentally deposited into highly vascularized keratin matrices (Bortolotti et al. 2008; Warne, Proudfoot, and Crespi 2015). Once fully grown, CORT and stable isotopes are trapped within the matrix and are believed to be stable over time. Collecting these less invasive samples can also be easily stored and do not require any special equipment (e.g., feathers can be stored at room temperature in envelopes). Therefore, keratin structures, here we emphasize feather, can capture average CORT levels that integrate both baseline and elevated secretion while concurrently capturing average dietary signatures over a much longer period. As described by Sergiel et al. (2017), combining concurrent measures of long-term stress and stable isotopes within the same study may open up a wide selection of testable ecological theories, including the relationship between CORT and diet.

Fairhurst et al. (2013; 2015) studied the synchronized deposition of CORT, δ13C, and δ15N in Dupont's lark *Chersophilus duponti* and Leach's Storm-petrels *Oceanodroma leucorhoa*, respectively, and concluded that combining CORT and stable isotopes may be a promising approach to provide complementary data on avian response to environmental conditions. However, the researchers went on to describe concerns about temporal asynchrony given that they used different feathers to measure each biomarker. In Fairhurst et al. (2013), the feather sample used for stable isotopes was a small piece of the feather (~1 mm^2) that was cut from the distal vanes of feathers used for CORT extraction. While in Fairhurst et al. (2015), feathers different from those used in CORT assays were analyzed for stable isotopes. We agree with the concern for temporal asynchrony given that studies have shown variation in CORT along a longitudinal axis of feather growth (Jenni-Eiermann et al. 2015), between feather types (e.g., body versus tail feathers, (Häffelin et al. 2021)) and melanization (Jenni-Eiermann et al. 2015). It is possible that assessing CORT levels and stable isotopes from a divided feather may not truly reflect how individuals are responding to their ecosystems but measuring both biomarkers from the exact same pool of feathers may get closer to describing an accurate response.

Here, we tested whether feathers that underwent the CORT extraction process can later be used to reliably measure $\delta^{13}C$ and $\delta^{15}N$. To do this, we utilized a single homogeneous pool of feathers to estimate HPA axis activity and $\delta^{13}C$ and $\delta^{15}N$ diet, respectively. We argue that using a single homogenous pool of feathers can reduce the possibility of misrepresenting the ecological context in which wildlife species exist. Furthermore, if successful, this "one sample two biomarkers" technique can maximize biological information from limited sample sizes often associated with field ecology and can promote the collection less invasive sample types. For

CORT analysis, feathers are washed in methanol, pulverized into a fine power, then extracted with methanol (Santymire et al. 2021). CORT is suspended in the methanol supernatant, which is removed then analyzed. Often, the feather pellets left are discarded. For stable isotopes analysis (SIA), samples are washed in a 2:1 chloroform:ether rinse then cut into pieces, air dried and then cut into pieces (Cherel, Hobson, and Weimerskirch 2000).

We used feather samples collected from house sparrows (*Passer domesticus*) to test whether the methanol-based extraction of CORT affects the reliability of subsequent $\delta^{13}C$ and $\delta^{15}N$ measurements. We used feathers from a single individual to compare samples that underwent the methanol-extraction process to feathers that did not. We hypothesized that methanol-extraction process does not affect the $\delta^{13}C$ and $\delta^{15}N$ values. We made this hypothesis based on a similar study that showed no difference in brown bear hair CORT and stable isotope signatures that underwent a methanol or 2:1 (v/v) mixture of chloroform: methanol rinse and extraction (Sergiel et al. 2017). We then validated $\delta^{13}C$ and $\delta^{15}N$ results by measuring the dietary shift from juveniles' insect-rich diet to adults' grain-rich diet. We predicted that juveniles would have significantly higher $\delta^{15}N$ and lower $\delta^{13}C$ values than adults if the CORT extraction process did not affect stable isotope values.

3.3 Methods

3.3.1 Study species

We plucked feathers (n = 50 to 75 left breasts) from n = 15 house sparrow specimens stored in basic refrigerators at the Field Museum, Chicago IL to test the "one sample two biomarkers" technique. Additionally, we collected house sparrows from across the Chicago metropolitan area and Indiana to test whether we could discriminate between the diet of juveniles (n = 97) and adults (n = 107) (see chapter 4 and 5 for capturing and sample collection methods).

3.3.2 Steroid hormone extraction

A methanol-based extraction was used to quantify steroid hormone levels, which was modified from Bortolotti and colleagues (2009). Whole feathers were first washed in 5 mL of 90% methanol in plastic tubes shaken on a large capacity mixer (Glas-Col, Terre Haute, IN) for 1 minute on setting 50. Tubes were then centrifuged for 2 minutes at 1500 rpm before pouring off the methanol. Wash was repeated two more times. Feathers were then placed into individual pre-labeled containers to allow for drying for 2-3 days at room temperature. Dry feathers (20.0 ± 0.5 mg) were weighed into 2mL microtubes with 2.8 mm ceramic beads (Omni International, Kennesaw, GA) and pulverized in the Omni Bead Ruptor 24 machine (Omni International, Kennesaw, GA) at 6.8m/s, 2x 45s, with 1x15s breaks. Then, 1 mL of 90% methanol was added to each sample and shaken for 4 hours at speed 50 on a large capacity mixer (Glas-Col, Terre Haute, IN). Samples were then centrifuged at 500x*g* for 25 minutes and the supernatant was poured off into a clean test tube. We dried the supernatant down under forced air in a hot water bath (60°C) until fully evaporated. Finally, we reconstituted the dried extract in 250 μL of phosphate-buffered saline at a 4x concentration. Reconstituted samples were sonicated for 20 minutes and then shaken on the large capacity mixer (speed 50) for 30 minutes. Samples were stored at -20°C until enzyme immunoassay (see chapter 4 CORT analysis). The remaining pulverized feather pellets in the microtubes were left under the fume hood to air dry over two days.

$CORT_f$ concentrations were analyzed using corticosterone enzyme immunoassays (EIA) using previously described methods (Munro and Stabenfeldt 1984; Santymire and Armstrong 2010). The corticosterone horseradish peroxidase (HRP) and polyclonal antiserum (CJM006) were provided by C. Munro (University of California, Davis, California, USA). The EIA was validated by demonstrating parallelism between binding inhibition curves of feather dilutions (8x conc., 4x conc., 2x conc., neat, 1:2, 1:4) and the corticosterone standard ($R^2 = 0.97$), and percent recovery (>80%) of exogenous corticosterone added to pooled feather samples ($y = 0.97x + 5.97$; $R^2 = 0.99$) using linear regression analysis. Assay sensitivity for corticosterone enzyme immunoassays was 1.95 pg/well and intra- and inter-assay coefficient of variation was <15% for all enzyme immunoassays.

3.3.3 Stable isotopes

We randomly divided house sparrow (n = 15) feathers into pair-wise treatments due to limited feather quantity. Treatments included: 1. methanol - feather pellets (0.02g ± 0.0005g and pulverized at 6.8m/s, 2x 45s, with 1x15s breaks) that remained after CORT hormone extraction (reported in Chapter 4); 2. non-methanol - feather pellets (0.02g ± 0.0005g and pulverized at 6.8m/s, 2x 45s, with 1x15s breaks) that did not undergo methanol extraction, and a control. The control represented feathers that were pulverized for one-ninth of the time of methanol and non-methanol (i.e., 1 round of 10 seconds) to ensure that stable isotope measurements did not reflect microtubes material we also pulverized feathers. It should be noted that we initially tried using scissors to produce feather pieces for the control but found that static reduced sample yield.

We used additional CORT-extracted feathers to test whether we could discriminate between the diet of juveniles (n = 97) and adults (n = 107). To investigate the dietary difference between juveniles and adults, we compiled average isotopic ratios for food items relevant to house sparrow diets to capture three categories: 1. Insects, 2. C3-enriched crops associated with conspicuously low $\delta^{13}C$ (e.g., sunflower seeds), and 3. C4-enriched crops associated with conspicuously high $\delta^{13}C$ (e.g., suet and cornmeal-based diet representing domestic chicken feed) (Table 3.1). Juveniles are provisioned a $\delta^{15}N$ enriched invertebrate diet by parenting adults, while adults themselves consume primarily grains, seeds, livestock feed that are either C3 enriched crops or C4 enriched crops (Gavett and Wakeley 1986a, 1986b; P.E. Lowther and Cink 1992). We used the fractionation (i.e., change in isotopic value relative to diet) values from a similar size bird (*Cyanistes caeruleus*) of 3‰ and 4‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively (Robb et al. 2011). We then calculated the consumption of food sources by adults and juveniles using a multi-source mixing model in the R package SIAR.

Table 3.1 Stable isotope value for potential food items. Fast-food beef and chicken represent a proxy for dietary information (e.g., livestock feed) that house sparrows would have access to during foraging.

Food Source	Mean d13C	SD d13C	Mean d15N	SD d15N	n	Reference
Insect	-25.95	2.099	5.43	1.95	114	a,b,c
Fast-food Beef	-18	2.9	6.5	0.4	162	d
Fast-food Chicken	-17.5	0.5	2.3	0.3	161	d
Corn	-11.9	1.7	4.6	1.62	2	d
Suet	-19.179	2.2775	1.874	0.7057	10	e
Sunflower	-27.657	0.694	2.3934	1.2741	7	e

[a] Ruhl et al. (2020), [b] Herrera et al. (2001), [c] Murray et al. 2015, [d] Jahren and Kraft (2008), [e] Hemert et al. (2012)

Each sample was weighed 0.5mg ± 0.005mg on a micro-analytical balance (Mettler Toledo, Columbus, Ohio) into 3.5 x 5 mm tin capsules (Costech Analytical Technologies INC, Valencia, CA) in duplicates. Samples were run in the Department of Geophysical Sciences at the University of Chicago, Chicago, Illinois. We determined the relative abundance of stable isotopes $\delta^{15}N$ and $\delta^{13}C$ by continuous-flow mass spectrometry using the Elemental Combustion System 4010 (Costech Analytical Technologies INC, Valencia, CA) with a ZeroBlank autosampler (Costech Analytical Technologies INC, Valencia, CA) connected to a Thermo Delta V Isotope Ratio Mass Spectrometer via a Conflo IV (Thermo Fisher Scientific INC, Waltham, MA). The system was controlled by Isodat software (Thermo Fisher Scientific INC, Waltham, MA) and the reported $\delta^{15}N$ and $\delta^{13}C$ values were corrected using an in-house working standard (glutamic acid-40/41 and Nestle Toll House cocoa powder).

3.4 Statistics

We analyzed normality using Shapiro-Wilk test. For normally distributed date ($p > 0.05$) we used an ANOVA to evaluate the "one sample two biomarker" hypothesis. For data that failed normality, we ran a Kruskal-Wallis non-parametric test. We then calculated Pearson correlation to assess the $\delta^{15}N$ and $\delta^{13}C$ relationship among feather treatments (i.e., methanol, non-methanol, and control). We expected samples from the same bird to be more alike than samples from others. Lastly, once normality was confirmed, we used a student's t-test to determine whether the $\delta^{13}C$ and $\delta^{15}N$ values were statistical different between juveniles and adults.

3.5 Results

The methanol-based method used to extract CORT from feathers did not affect $\delta^{13}C$ (Kruskal-Wallis $\chi^2 = 0.51$, df = 2, p-value = 0.77) and $\delta^{15}N$ values ($F_{2,42} = 0.022$, p-value = 0.97). Methanol and control treatments were positively correlated for both $\delta^{13}C$ (methanol v. non_methanol: $r = 0.99$ $p < 0.001$) and $\delta^{15}N$ (methanol v. control: $r = 0.89$ $p < 0.001$; Fig. 3.1a and b, respectively).

$\delta^{15}N$ values were significantly different between adults and juveniles ($p < 0.001$). Adult $\delta^{15}N$ values were lower (6.72 ± 0.069, n = 207) than juvenile δ^{15} N values (7.38 ± 0.087, n = 95). Similarly, $\delta^{13}C$ was also significantly different between the two age groups ($p < 0.001$). Here, adults (-16.33 ± 0.23, n = 207) have higher $\delta^{13}C$ than juveniles (-20.13 ± 0.21, n = 95). Using a biplot, we were able to visualize that the isotopic signatures for juveniles plotted closer to the average isotopic signature of insect while adults plotted closer to that of suet and chicken (a proxy for chicken feed) (Fig. 3.2)

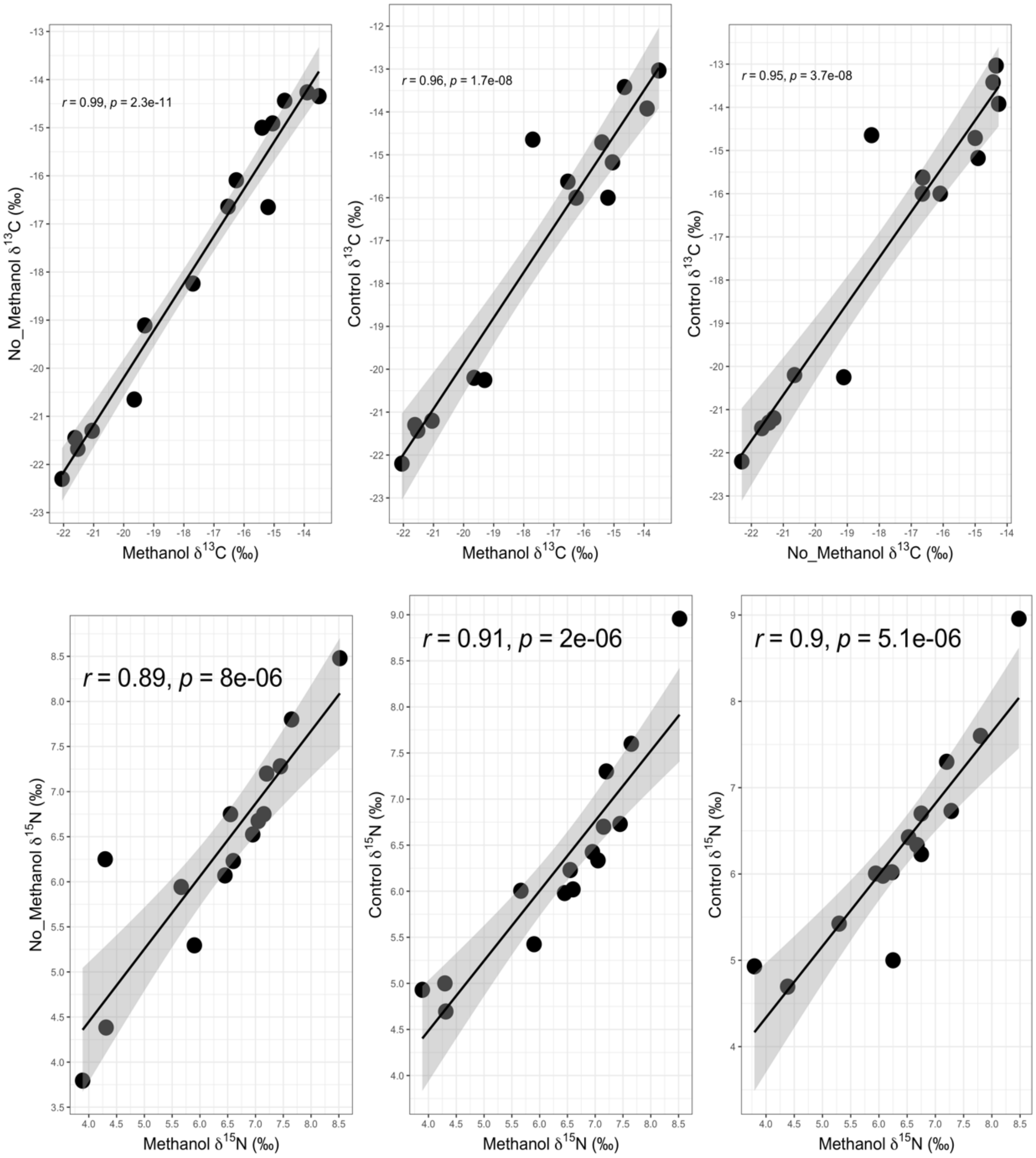


Figure 3.1 Scatterplot of stable isotope of (top) δ^{13}C ‰ and (bottom) δ^{15}N ‰ for house sparrow feather treatments (n = 15 per treatment) with 95% confidence interval.

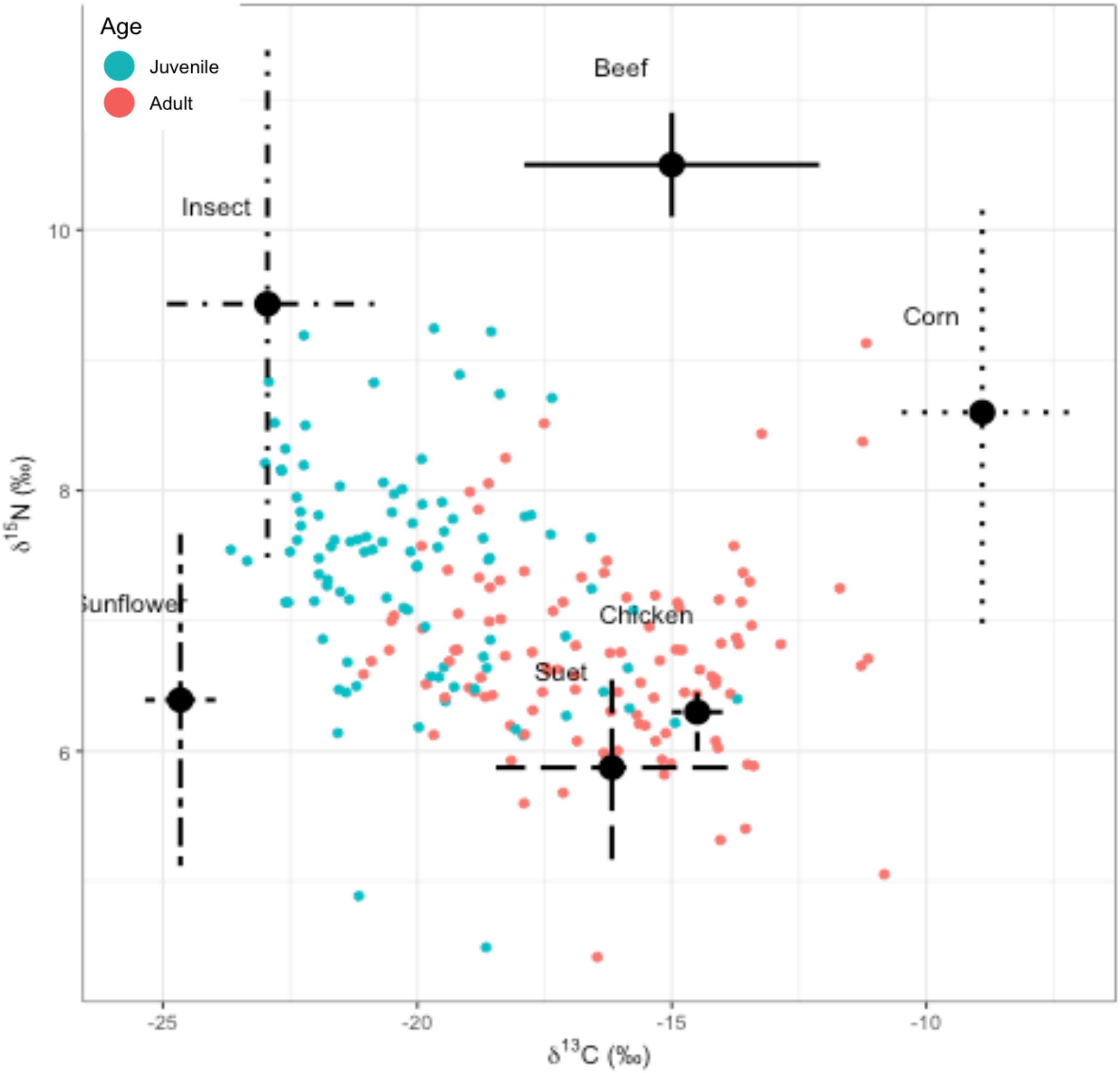


Figure 3.2 Biplot depicting mean and standard deviation of stable isotope food sources (e.g., insect, sunflower seed, suet, and corn), domesticated livestock products (e.g., chicken and beef). Isotopic ratios for house sparrow juveniles (n = 95) and adults (n = 107).

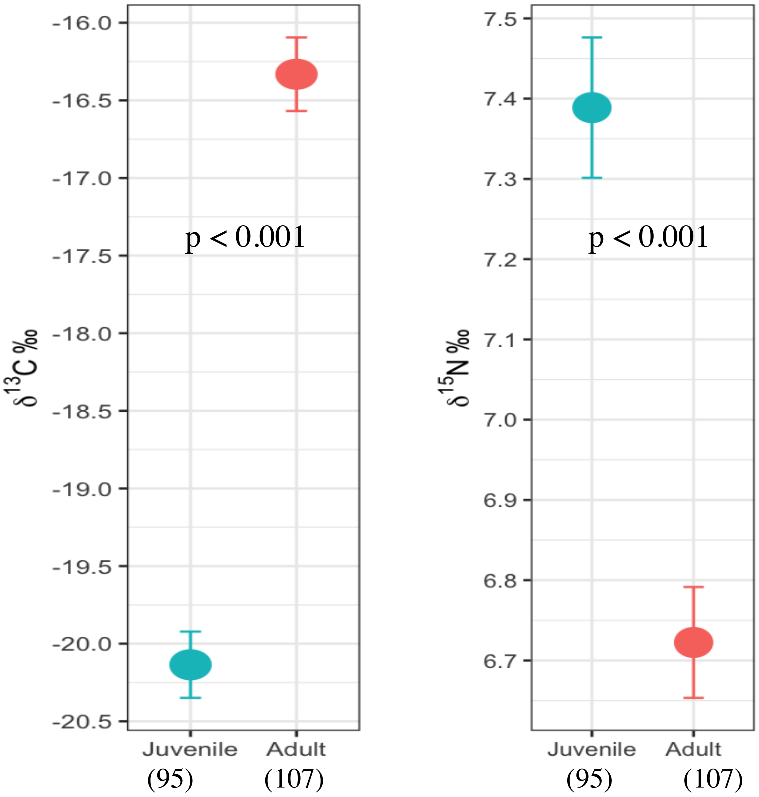


Figure 3.3 Juvenile and adult mean and standard error plotted for (left) $\delta^{13}C$ and (right) $\delta^{15}N$. Values below represent sample size.

3.6 Discussion

We found a strong positive correlation for $\delta^{13}C$ and $\delta^{15}N$ values between CORT-extracted feathers, i.e., methanol treatment, and feathers that were not treated with methanol, non-methanol treatment, and the control. These results support our hypothesis that the CORT-extraction process does not affect the stable isotope ratios for carbon and nitrogen. Support for these findings was found in (Bontempo et al. 2014). Bontempo and colleagues were able to demonstrate that stable isotopic values for $\delta^{13}C$ and $\delta^{15}N$ were not affected by cleaning mixtures (chloroform: methanol 2:1 and diethylether:methanol 2:1).

We were also able to support the reliability of measuring $\delta^{13}C$ and $\delta^{15}N$ from CORT-extracted feathers. To do this we provided evidence that showed the trophic structure of juveniles and adults via the shift from an insect-based diet to a majority plant-based diet. Our study confirms the reliability that CORT extracted feathers can reliably be used to quantify $\delta^{13}C$ and $\delta^{15}N$ values. The age-related distinction between $\delta^{13}C$ and $\delta^{15}N$ (Fig. 3.3) is consistent with those captured by Hodum and Hobson (2000), who found higher mean $\delta^{15}N$ and lower mean $\delta^{13}C$ in the blood of three species of petrel chicks when compared to their adult conspecifics (*Fulmarus glacialoides, Thalassoica antarctica, Daption capense, and Pagodroma nivea*) that resembled the shift from a fish-based diet in chicks to a zooplankton-based diet in adults.

Ultimately, using the "one sample two biomarkers" technique can reduce concerns about temporal asynchrony associated with using different sample types and different sections (as seen in Fairhurst et al. (2013, 2015)) to estimate average CORT levels and dietary signature. The technique may better reflect how free-living individuals are responding to their ecosystems because (theoretically) CORT and stable isotopes are deposited during feather and hair growth, which may effectively advance urban ecology.

Limited sample volume does not need to equate to limited information. Our results support the opportunity for urban ecologists, particularly those studying free-living animals, to maximize ecological information on diet and steroid hormone levels. We speculate too, that much like the feathers used in our experiment, fur and hair may yield similar comparable results after a methanol-based steroid extraction. Furthermore, the "one sample two biomarkers" technique can save researchers a considerable amount of time in the laboratory. Researchers can run a single washing and pulverizing session instead of repeating these processes twice (Sergiel et al. 2017). Reduced sample preparation time in the laboratory can provide opportunities to pursue additional interests. Additionally, the technique may have trickling effects such as reducing waste (e.g., plastic tubes, solutions, and gloves) and energy expenditure (e.g., electricity), thus improving the ecological footprint generated by scientific laboratories. The technique may also impact the overall laboratory expense by reducing the number of purchases made to restock supplies.

Although CORT and stable isotopic signatures deposited into feathers can provide insight into how individuals are responding to their environment, there are still two noteworthy limitations. We currently assume that (1) glucocorticoids are deposited into the keratin matrix via passive diffusion from circulating blood during the period of feather/hair growth alone and (2) glucocorticoid molecules remain trapped within the keratin structure and therefore stable once deposited. Studies have yet to directly test these assumptions. However, taken largely from studies that examined cortisol concentrations in hair, there is reason to believe that cortisol could be deposited outside of passive diffusion and that concentrations may even change within minutes. For instance, cortisol levels were lowest among brown bears *Ursus arctos* that experienced less stressful hair collection methods when compared to individuals that experience more stressful hair collection methods (Cattet et al. 2014). Results from Cattet et al. (2014) showed that cortisol concentration in hair quickly increased in response to capture and handling techniques, suggesting that glucocorticoids may be deposited into keratin structures even after growth. Furthermore, there are no studies to demonstrate the molecular interaction between glucocorticoids within keratin matrices and thus its stability over time remains in question. We suggest that future researchers should conduct experiments that directly test these two assumptions. Until future validation is provided, the "one sample two biomarkers" technique may reduce temporal asynchrony when interpretating the relationship between CORT and stable isotope values, which may capture a more meaningful picture for how CORT and diet change in sync to environmental opportunities and challenges in wildlife ecosystems.

Chapter 4

Free-living house sparrows along an urbanized gradient differ by body condition and diet, but not their glucocorticoid levels.

4.1 Abstract

Urbanization and its marked increase in human presence will continue to expose wildlife to unique environmental challenges and opportunities. Access to anthropogenic foods can help to buffer the effects of seasonal food shortages, but such foods may not meet the nutritional requirements needed for wildlife health. Similarly, corticosterone, the primary glucocorticoid in birds, is released in response to environmental stressors can be adaptive, however detrimental effects can occur if its magnitude and duration surpass a range that can compromise fitness. In this study, we aimed to explore the effect of diet, corticosterone, and their bidirectional relationship on body condition of house sparrows along an urban gradient. Corticosterone and diet have been linked to the body condition of many species including birds. Studying the interaction between corticosterone levels and diet could help determine how wild birds are responding to stressors and diet quality concurrently. We measured corticosterone, the primary glucocorticoid in birds, and isotopic signatures of diet ($\delta^{13}C$ and $\delta^{15}N$) using feathers. Feathers represent a long-term integrated average for the concurrent deposition of corticosterone, $\delta^{13}C$, and $\delta^{15}N$, which can provide a better understanding for how house sparrows are interacting and reacting to their urban-rural habitats. We hypothesized that corticosterone and diet work in sync to alter body condition, and that the relationship may differ along an urbanized gradient. Our results indicated that corticosterone did not affect house sparrow body condition. We found no

evidence to suggest that corticosterone interacts with diet in a meaningful way to affect body condition. However, our results do show that individuals living in the most urban habitat had significantly higher body condition and mass than those living in the least urbanized habitat. We then detected a negative correlation between body condition and $\delta^{13}C$, wherein birds with higher $\delta^{13}C$ values had significantly lower body conditions. These results underscore that urban habitat can be beneficial while challenging the hypothesis that urbanization leads to lower body condition and mass.

4.2 Introduction

Urbanization has transformed habitats leading to greater pollution levels (e.g., light, chemical, and noise), altered flora and fauna biodiversity, higher anthropogenic food resources, and warmer temperatures (Arnfield 2003) when compared to less urbanized areas. Although urbanization is noted as a significant threat to wildlife, there is no evidence for it coming to a halt. Instead, the United Nations predicts that by 2030, six out of every 10 people will be city dwellers and that will rise to seven out of every 10 people by 2050 (United Nations 2018). The marked increase in human presence will continue to expose wildlife to unique environmental challenges and opportunities. However, advantageously, species have evolved integrated physiological responses to cope with the changing environments in which they live. Monitoring endogenous responses of free-living birds is therefore critical to comprehend the processes and limitations that influence their life-history traits (e.g., reproduction and growth) under novel environmental perturbations such as those resulting from the Anthropocene, particularly urbanization.

The HPA axis chemical cascade begins with a stressor that activates the release of corticotropin releasing hormone (CRH) from the hypothalamus. CRH then targets the pituitary gland, which triggers the release of adrenocorticotrophic hormone (ACTH). Finally, an animal's physiological and behavior is altered to respond to the stressor with the release of CORT from the adrenal gland. Once the stressor is alleviated CORT release stops via a negative feedback loop. Although the short-term elevation of CORT is considered adaptive, it is the long-term elevation of CORT that may have detrimental consequences on body condition, reproduction, and growth (Exton 1972; Dallman et al. 1993; Lattin and Romero 2015).

Stable isotope ratios can provide isotopic signatures to estimate diet choices. Stable isotopes carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$), which are denoted as $\delta^{13}C$ and $\delta^{15}N$, respectively, have been applied to assess the dietary information of wild birds and mammals since the late 1970s (reviewed in Kelly (2000)). Carbon provides information on the isotopic signature that reflects the metabolic differences between C4 plants (e.g., corn, sugarcane) and C3 plants (e.g., cereal grains, sunflower), which leads to an enriched $\delta^{13}C$ in C4 plants when compared to C3 plants. Carbon stable isotopes ratios are currently being used to determine whether wildlife animals are consuming more anthropogenic foods or natural foods (Newsome et al. 2010; Penick, Savage, and Dunn 2015). $\delta^{15}N$ is useful to infer trophic levels. As trophic level moves from the base of a food chain to top predators, $\delta^{15}N$ is increasingly enriched in the tissue, which results in plants having lower $\delta^{15}N$ than herbivores (e.g., predatory insects), and herbivores having lower $\delta^{15}N$ than omnivores (e.g., birds) and carnivores (e.g., wolves) (DeNiro and Epstein 1981). Carbon provides information on the isotopic signature that reflects the metabolic differences between C4 plants (e.g., corn, sugarcane) and C3 plants (e.g., cereal grains, sunflower), which leads to an enriched $\delta^{13}C$ in C4 plants when compared to C3 plants.

Because North American diets tend to have a higher proportion of C4-derived foods, the $\delta^{13}C$ values can be used to determine whether birds are consuming anthropogenic food resources along an urbanized gradient, and to what extent do they rely on these resources for food (Jahren and Kraft 2008). On the other hand, $\delta^{15}N$ is useful to infer trophic levels. As trophic level moves from the base of a food chain to top predators, $\delta^{15}N$ is increasingly enriched in the tissue, which results in plants having lower $\delta^{15}N$ than herbivores (e.g., predatory insects), and herbivores having lower $\delta^{15}N$ than omnivores (e.g., birds) and carnivores (e.g., wolves) (DeNiro and Epstein 1981).

Measuring the interdependent relationship between an organism's glucocorticoid modulation and food resources may help scientists to better understand how organisms are coping with this ever-expanding threat (Astheimer, Buttemer, and Wingfield 1992; Kitaysky et al. 2001; Cote et al. 2006; Schoech et al. 2007; Warne, Proudfoot, and Crespi 2015). To date, we have some understanding of how food resources and glucocorticoids independently contribute to avian health and adaptation, but there is still much work yet to be done for understanding how avian glucocorticoids and available food resources respond in concert to their urbanizing environments. As suggested by Warne and colleagues (2015), it is the integration of the two that is necessary to contextualize and interpret whole-organism adaptive, plasticity, and fitness capability.

Studies on corticosterone (hereafter CORT, the primary glucocorticoid in birds), and food resources in vertebrates have shown evidence of being tailored by the unique environments in which they live. One major takeaway is that limited access to nutritional foods and a maladaptive CORT response can result in deleterious effects on individuals via decline in body condition, reproductive performance, and survival (Kitaysky, Wingfield, and Piatt 2001; Kitaysky et al.

2003; Kitaysky, Piatt, and Wingfield 2007; Bauer et al. 2011). For instance, CORT levels can vary along urban gradients (Bókony et al. 2021), and so too can available food resources (Guiry and Buckley 2018). Urbanization can have an effect on both baseline CORT and stress-induced CORT; however, the trend is not uniformed (reviewed in Bonier (2012)). Birds in urbanized areas have shown lower, higher, or no difference in both baseline and stress-induced CORT levels when compared to their rural conspecifics. As for food resources, animals in more urbanized areas are expected to have greater access to anthropogenic foods from human garbage and/or feeders, which may buffer seasonal changes in foods (Chace and Walsh 2006; Bernat-Ponce et al. 2021). Urbanization can also reduce natural food availability (e.g., insect density) thus limiting the nutritional value required to carry out biological functions (Pollock et al. 2017).

The relationship between CORT and diet is also bidirectional and can have indirect effects on animal health. For instance, food deprivation and foods with low nutritional quality can induce CORT secretion (Astheimer, Buttemer, and Wingfield 1992; Jenni-Eiermann et al. 2008); while CORT itself can promote feeding behaviors, such as increased foraging and begging (Astheimer, Buttemer, and Wingfield 1992; Cote et al. 2006; Wada and Breuner 2008). These physiological states can then impact an animal's energy reserves, which is assumed to relay information about fitness since it is indicative of an individual's health and condition (Peig and Green 2009, 2010). Liker et al. (2008) showed that house sparrows (*Passer domesticus*) in urbanized habitats had consistently lower body mass and were in worse condition than those in rural areas. Likewise, there is evidence of decreased fat reserves in urban blackbirds, *Turdus merula,* and lower body mass in urban rufous-collared sparrows, *Zonotrichia capensis,* when compared to their rural-dwelling conspecifics (Ruiz et al. 2002; Partecke, Schwabl, and Gwinner 2006). These results suggest that urban diets may not meet the energy requirements for daily and seasonal life-history demands, and thus are leading to poorer body conditions when compared to less urbanized birds. However, these differences are not applicable to all urbanized gradients as other studies have shown no difference in body condition of house sparrows (Bókony, Kulcsár, et al. 2012; Gadau et al. 2019).

In this study, we sought to better understand how CORT levels and diet influence the body condition of a well-known human-commensal, the house sparrow. Though native to parts of Asia, North Africa, and most of Europe, house sparrows are considered urban-exploiters because their expansion that began ~10,000 years ago has led to a global distribution, which is closely associated with the spread of human agriculture and urbanization (Summers-Smith 2003; Anderson 2006; Hanson et al. 2020). They have maintained an ecological edge by coupling modulation of CORT production such as downregulating their CORT reactivity (Lendvai, Bókony, and Chastel 2011), reduced neophobia (Bókony, Kulcsár, et al. 2012), and their willingness to explore novel food resources (2005). For the most part, where there are humans, there are these anthro-dependent sparrows. Although still widespread, global populations are beginning to decline in some areas (e.g., Europe (Summers-Smith 2003), Asia (Dandapat, Banerjee, and Chakraborty 2010), and North America (P. Lowther, Cink, and Billerman 2020)), but the primary cause is still unclear. These declines are also widespread throughout the United States including Illinois and Indiana (Fig. 4.1 and 4.2). Studies have speculated global declines are due to reduced insect availability for nestlings (Seress et al. 2012), increased pollution (Herrera-Duenas et al. 2014), and infectious diseases (Dadam et al. 2019). Given their widespread abundance along an urbanized gradient and the interest in what is causing their ongoing decline, house sparrows represent an ideal study model to assess the processes and limitations of urbanization on avian CORT levels and available food resources.

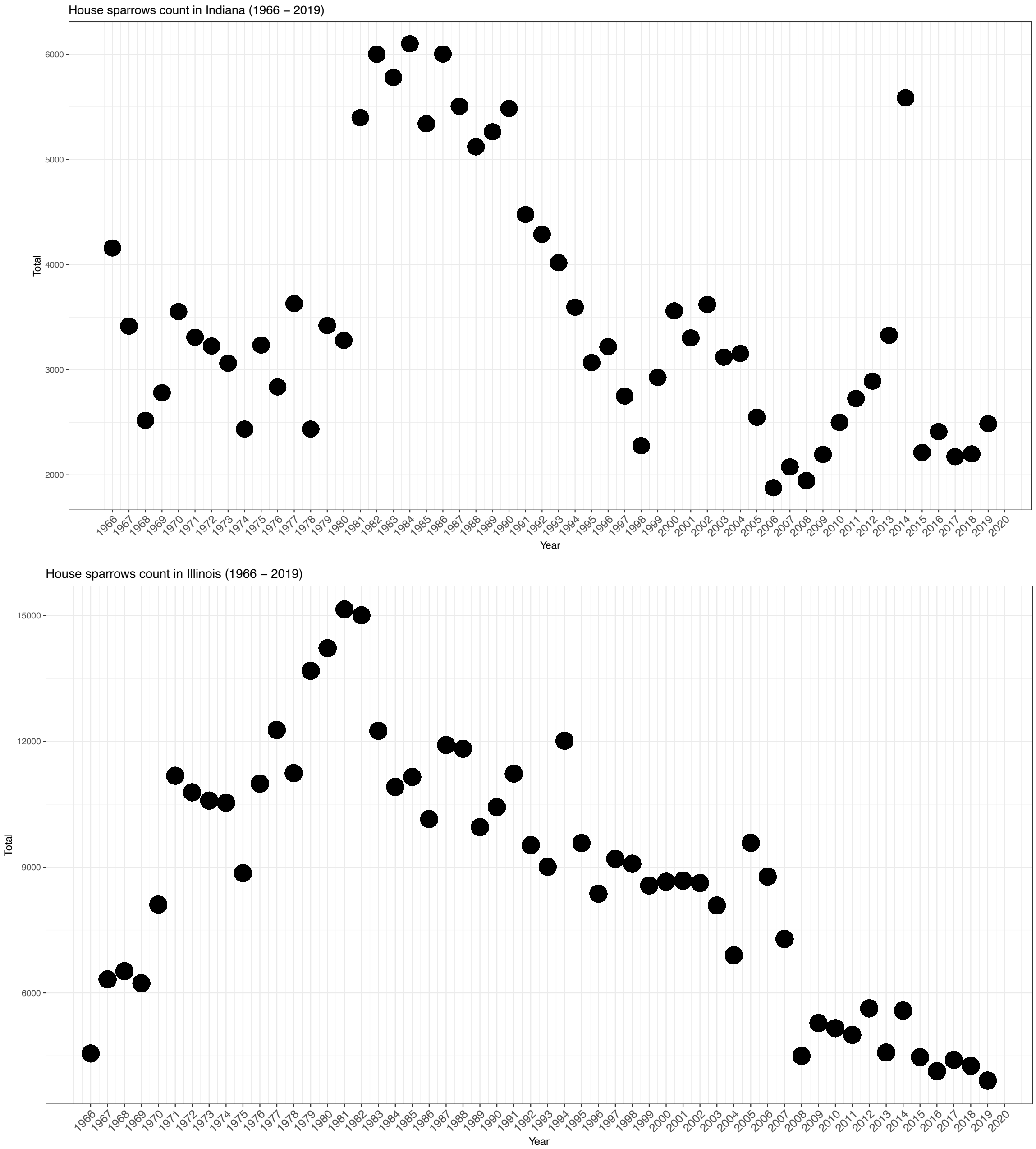


Figure 4.1 (top) Indiana and (bottom) Illinois house sparrow population count from 1966 to 2019. Raw data from 2020 Release - North American Breeding Bird Survey Dataset (1966-2019) from United States Geological Survey.

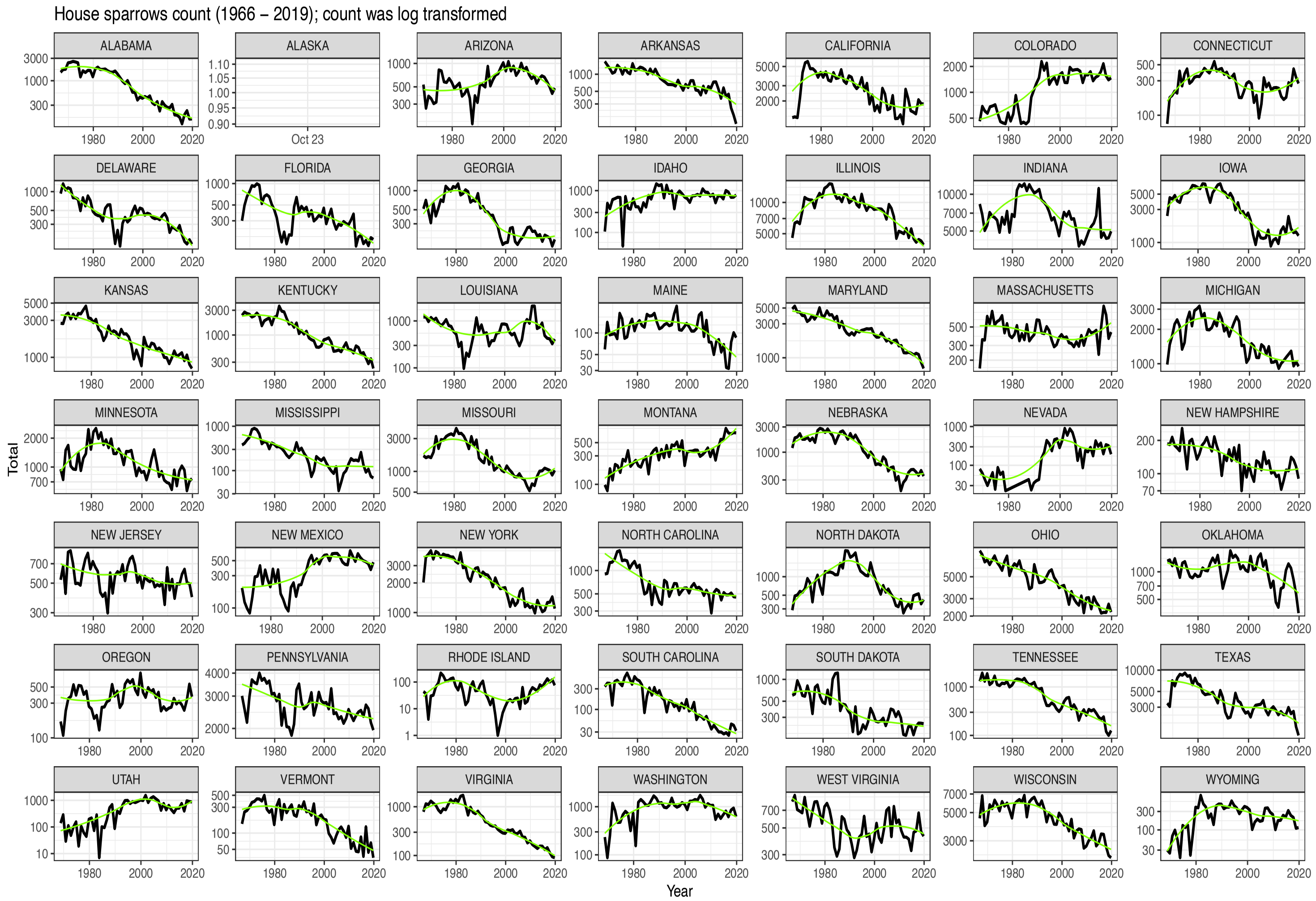


Figure 4.2 House sparrow population count across 49 USA states from 1966 to 2019. Raw data from 2020 Release - North American Breeding Bird Survey Dataset (1966-2019) from United States Geological Survey.

Our goal is to explore the relationship between CORT and diet and their effect on the body condition of free-living house sparrows using the "one sample two biomarkers" technique described in Chapter 3. We hypothesized that CORT and diet work in sync to alter body conditions along an urbanized gradient. We predict that CORT and diet will be significantly correlated, however the direction of that correlation we withdraw from assuming given the heterogeneity in reported results. We speculate for instance, that if urban birds have the poorest body condition, it may be associated with increased access to (low quality) anthropogenic foods and/or high CORT levels. We also investigated the role that life history stages (e.g., age, breeding season) may have on CORT levels and stable isotope values as the associations have been documented in previous studies (Cherel, Hobson, and Weimerskirch 2000; Lattin et al. 2012; Warne, Proudfoot, and Crespi 2015; L. Romero et al. 2017).

4.3 Methods

4.3.1 Study species and sites

House sparrows (n = 97) were captured from three sites along an urbanized gradient in the midwestern (Illinois and Indiana) United States from May 2017 to June 2019. A gradient from least to most urban was calculated based on the housing units per hectare, a proxy for human population density (modification from Bierwagen et al. (2010)). We extracted the total number of housing units within a 2 km buffer of each site (the home range of house sparrows, (P.E. Lowther and Cink 1992)) using University of Wisconsin-Madison SILVAS Lab 2010 shapefiles. We then calculated housing units per hectare (HUH) and found that our study sites were different ($\chi^2 = 52$, df = 2, $p < 0.0001$) with Lincoln Park Zoo (LPZ), in Chicago, IL, USA (273.47 HUH) had the highest human population followed by Homewood, IL, USA (30.97

HUH) and Hebron, IN, USA (2.58 HUH), respectively. The two closest locations (LPZ and Homewood) were ~47 km apart and with a home range of 2km, it was therefore unlikely that individual house sparrows moved from one habitat to another. We used a combination of mist nests and Tomahawk live-traps baited with sunflower seeds to collect juveniles (n = 30), adults (n = 67). Upon capture, we recorded age and sex and collected body feathers (n = ~25 feathers) from the left breast of each individual. Feathers were then stored in opaque envelopes at room temperature. Each house sparrow was then fitted with a U.S. Geological Survey aluminum leg band (permit# 09924). All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee (ACUP # 72510).

4.3.2 Stable isotopes

Homogenized feather samples were weighed 0.5mg ± 0.005mg on a micro-analytical balance (Mettler Toledo, Columbus, Ohio) into 3.5 x 5 mm tin capsules (Costech Analytical Technologies INC, Valencia, CA) in duplicates. Samples were run in the Department of Geophysical Sciences at the University of Chicago, Chicago, Illinois. We determined the relative abundance of stable isotopes $\delta^{15}N$ and $\delta^{13}C$ by continuous-flow mass spectrometry using the Elemental Combustion System 4010 (Costech Analytical Technologies INC, Valencia, CA) with a ZeroBlank autosampler (Costech Analytical Technologies INC, Valencia, CA) connected to a Thermo Delta V Isotope Ratio Mass Spectrometer via a Conflo IV (Thermo Fisher Scientific INC, Waltham, MA). The system was controlled by Isodat software (Thermo Fisher Scientific INC, Waltham, MA) and the reported $\delta^{15}N$ and $\delta^{13}C$ values were corrected using an in-house working standard (glutamic acid-40/41 and Nestle Toll House cocoa powder). Samples outside of the experiment were processed identically.

4.3.3 Steroid hormone extraction

A methanol-based extraction was used to quantify steroid hormone levels, which was modified from Bortolotti and colleagues (2009). Whole feathers were first washed in 5 mL of 90% methanol in plastic tubes shaken on a large capacity mixer (Glas-Col, Terre Haute, IN) for 1 minute on setting 50. Tubes were then centrifuged for 2 minutes at 1500 rpm before pouring off the methanol. Wash was repeated two more times. Feathers were then placed into individual pre-labeled containers to allow for drying for 2-3 days at room temperature. Dry feathers (20.0 ± 0.5 mg) were weighed into 2mL microtubes with 2.8 mm ceramic beads (Omni International, Kennesaw, GA) and pulverized in the Omni Bead Ruptor 24 machine (Omni International, Kennesaw, GA) at 6.8m/s, 2x 45s, with 1x15s breaks. Then, 1 mL of 90% methanol was added to each sample and shaken for 4 hours at speed 50 on a large capacity mixer (Glas-Col, Terre Haute, IN). Samples were then centrifuged at 500x*g* for 25 minutes and the supernatant was poured off into a clean test tube. We dried the supernatant down under forced air in a hot water bath (60°C) until fully evaporated. Finally, we reconstituted the dried extract in 250 μL of phosphate-buffered saline at a 4x concentration. Reconstituted samples were sonicated for 20 minutes and then shaken on the large capacity mixer (speed 50) for 30 minutes. Samples were stored at -20°C until enzyme immunoassay.

The remaining pulverized feather pellets in the microtubes were left under the fume hood to air dry over two days. Samples were then stored at room temperature until the stable isotope analysis (see Chapter 3 for methodology).

CORT_f concentrations were analyzed using corticosterone enzyme immunoassays (EIA) using previously described methods (Munro and Stabenfeldt 1984; Santymire and Armstrong 2010). The corticosterone horseradish peroxidase (HRP) and polyclonal antiserum (CJM006) were provided by C. Munro (University of California, Davis, California, USA). The EIA was validated by demonstrating parallelism between binding inhibition curves of feather dilutions (8x conc., 4x conc., 2x conc., neat, 1:2, 1:4) and the corticosterone standard ($R^2 = 0.97$), and percent recovery (>80%) of exogenous corticosterone added to pooled feather samples ($y = 0.97x + 5.97$; $R^2 = 0.99$) using linear regression analysis. Assay sensitivity for corticosterone enzyme immunoassays was 1.95 pg/well and intra- and inter-assay coefficient of variation was <15% for all enzyme immunoassays.

4.3.4 Scaled mass index

To calculate the body condition of each individual using the "scaled mass index" (hereafter SMI, (Peig and Green 2009, 2010)), we measured the beak, (0.00mm to 150.00 mm Mitutoyo Absolute AOS Digimatic; Model No. CD-G" ASX), and body mass (to the nearest 0.1g using Pesola Marque déposée 0.0g to 50.0g) The SMI was calculated for adults alone because of limited length and body mass measurements for juveniles.

$$SMI_i = M_i\, x\, (L_o/L_i)^{b_{SMA}}$$

The SMI adjusts the mass of each individual to the expected mass if they had the same body size, where M_i and L_i are the body mass and the beak length of the individual i, respectively, L_0 the arithmetic mean ($L_0 = 12.31$ mm, n = 53 adults) of the beak length for the population and the slope (*b* = 0.77*)* estimate of a standardized major axis (SMA) regression of log-transformed body mass on log-transformed beak length. We used beak length instead of tarsus length to calculate SMI because it best correlated with body mass (beak length: $r = .37$, $p = 0.006$; tarsus length: $r = 0.12$, $p = 0.38$).

4.4 Statistical analyses

We used δ13C to capture the diet of adults in all downstream analyses due to the limited sample size ($n = 53$ adults). We reasoned that given the life history and foraging behavior of adult sparrows, δ13C is more indicative of their diet than δ15N.

We used the Kruskal-Wallis nonparametric test to identify differences between sex, breeding season, and location for $\delta^{13}C$ and $CORT_f$ in adults, followed by the Wilcoxon test to assess biological consistency between groups because data were not normally distributed including after transformation. We used Pearson's correlation to identity relationships between main effects (e.g., $CORT_f$ and $\delta^{13}C$). We treated location as a categorical factor. Finally, the effect of $CORT_f$ and diet, and their interaction on the SMI of adults were analyzed using a general linear mixed model (GLMM) with the breeding season as the random factor to account for seasonal variation (breeding: spring to summer and non-breeding: fall to winter). We tested for normality using a Shapiro-Wilk test and determined that log-transformed SMI improved normality ($W = 0.99$, $p = 0.86$). The variance explained by the model was calculated using the marginal R^2 (variance explained by the fixed factors) and conditional R^2 (variance explained by both fixed and random factors). We used an ANOVA to compare the mean log-SMI (log transformed to a normal distribution) among locations and pairwise TukeyHSD for comparisons between locations. Juveniles were excluded from the SMI analysis altogether because data did not exceed n = 8 individuals. All statistical analyses were performed in R Version 1.1.456 (R Core Team, 2020).

4.5 Results

4.5.1 Feather corticosterone

Juveniles (5.64 ± 0.36 ng/g) had higher overall CORT$_f$ levels than adults (CORT$_f$ 2.17 ± 0.13 ng/g) ($\chi^2 = 44.001$, df = 1, $p < 0.0001$, Fig. 4.3). Higher CORT$_f$ in juveniles was also observed within the LPZ ($p < 0.0001$, adults: 1.83 ± 0.16 ng/g, juveniles: 6.18 ± 0.670 ng/g) and Homewood site ($p < 0.0001$, adult, 2.73 ± 0.29 ng/g; juveniles, 5.37 ± 0.426 ng/g) relative to the Hebron site. Both season ($\chi^2 = 0.031$, df = 1, p= 0.86; breeding: n = 30, non-breeding: n = 34) and sex ($\chi^2 = 0.11$, df = 1, p = 0.7421; n = 25 females, n = 27 males) were not associated with adult house sparrow CORT$_f$ concentrations. However, within the non-breeding season, adult sparrows at Homewood (p = 0.039; 2.99 ± 0.33 ng/g) had higher CORT$_f$ than those at Hebron ($p < 0.001$; 1.98 ± 0.22 ng/g) and LPZ (1.59 ± 0.25 ng/g).

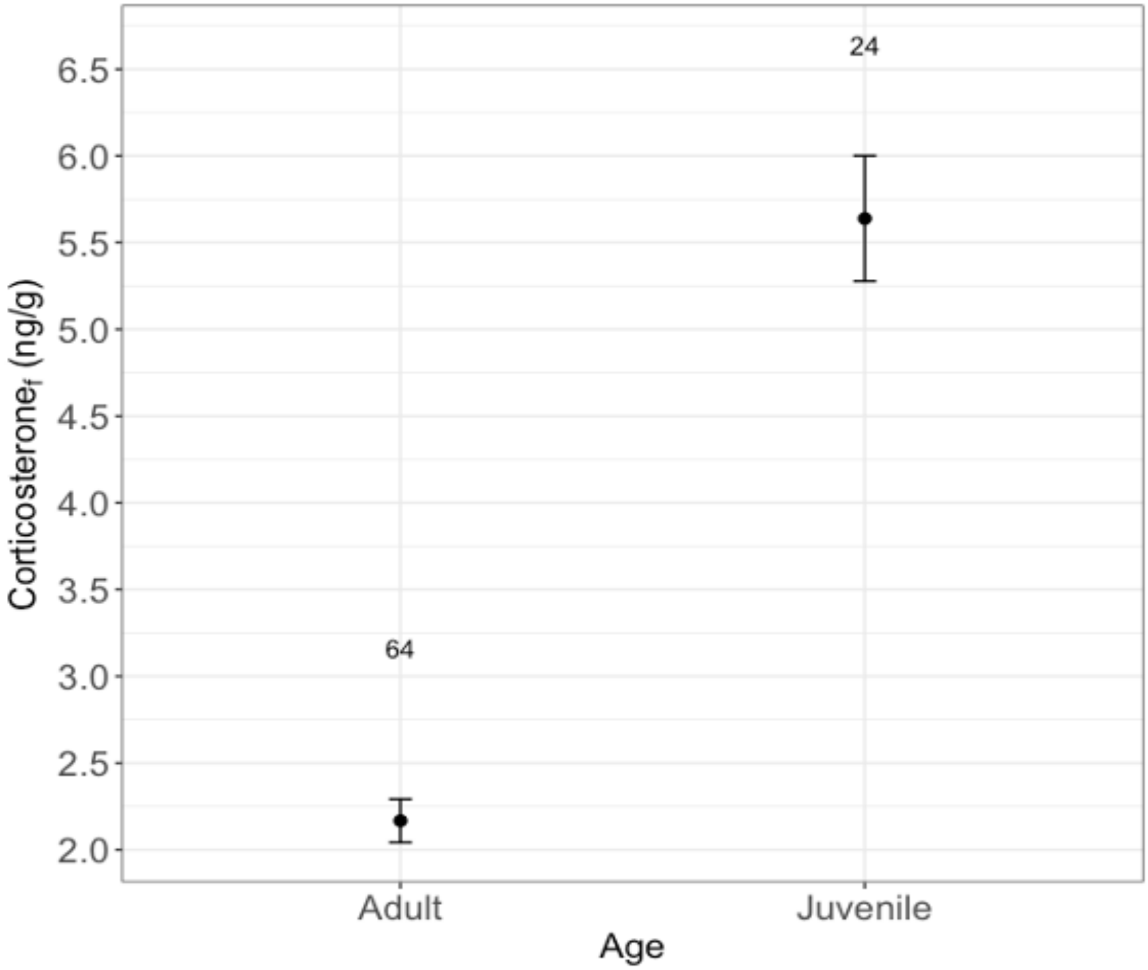


Figure 4.3 Mean (± SE) feather corticosterone in age juvenile and adult house sparrows. The asterisk indicates a difference ($p < 0.05$) between age class. Numbers represent sample size.

4.5.2 Stable isotopes

As reported in Chapter 3, juveniles (mean ± se: -20.93 ± 0.46 ‰, n = 25) had lower ($\chi2$ = 37.486, df = 1, p < 0.0001) δ13C values than adults (-16.14 ± 0.37 ‰, n = 62), but had higher ($\chi2$ = 19.472, df = 1, p < 0.0001) δ15N values (7.89 ± 0.22 ‰, n = 25) than adults (7.023 ± 0.095 ‰, n = 57). When looking solely at adults, total δ13C tended to be higher ($\chi2$= 3.53, df = 1, p = 0.06) in the non-breeding season (-15.47 ± 0.40‰, n = 31) compared to the breeding season (-16.80 ± 0.60‰, n = 31). However, within each location δ13C was similar (all p > 0.05 when adjusted by false discovery rate (fdr); Fig. 4.7.2) between the breeding and non-breeding seasons. δ15N values for adults were significantly ($\chi2$ = 4.671, df = 1, p-value = 0.030) higher in the breeding season (7.44 ± 0.14 ‰, n = 51) when compared to the non-breeding season (7.01 ± 0.11 ‰, n = 31). A significant difference between breeding versus non-breeding was not observed within each location (p > 0.05 when adjusted by fdr; Fig. 4.7.3). Within adults, δ13C also did not differ by sex (t = 0.10, df = 48, p-value = 0.91; female: -16.31 ± 0.58, n = 25; male: -16.40 ± 0.65, n = 25). Within adults, δ15N differed significantly for males and females in the Hebron site ($\chi2$ = 7.0848, df = 1, p-value = 0.01; in Fig. 4.7.4). Females (7.81 ± 0.31 ‰, n = 7) had significantly higher δ15N than males (6.54 ± 0.26 ‰, n = 8).

4.5.3 Scaled Mass Index

We determined that body mass (t_{51} = 0.84, p = 0.40) and beak (t_{51} = 0.17, p = 0.87) were similar between male and female adults; therefore, we combined them for the SMI analyses. We found that log-SMI varied ($F_{2,50}$ = 13.68; p < 0.0001) along the urbanized gradient (Fig. 4.4).

Specifically, adult house sparrows living in the most urbanized area (LPZ; 32.0 ± 2.94) had higher ($p < 0.0001$, $q = 3.42$) SMI than those living in the least urbanized area (Hebron; 28.1 ± 1.98); however, SMI was similar between LPZ ($p = 0.24$, $q = 3.42$) and the intermediate urbanized area (Homewood; 29.4 ± 2.90), and between Hebron and Homewood ($p = 0.67$, $q = 3.42$). The results from the GLMM showed that the fixed factors ($\delta^{13}C$, $CORT_f$) contributed to 30% of the variation in adult log-SMI, with $\delta^{13}C$ being the only significant factor ($t_{46} = -3.16$, $p = 0.0025$; Table 4.1). The interaction between δ13C and $CORT_f$ was nearly significant ($t_{46} = 0.0076$, $p = 0.074$; Table 4.1). The variance explained by the random effect amounted to 0%. The results from the Pearson's correlation for the relationship between $\delta^{13}C$ and log-SMI showed a negative correlation ($r = -0.49$, $p < 0.001$; Fig. 4.5).

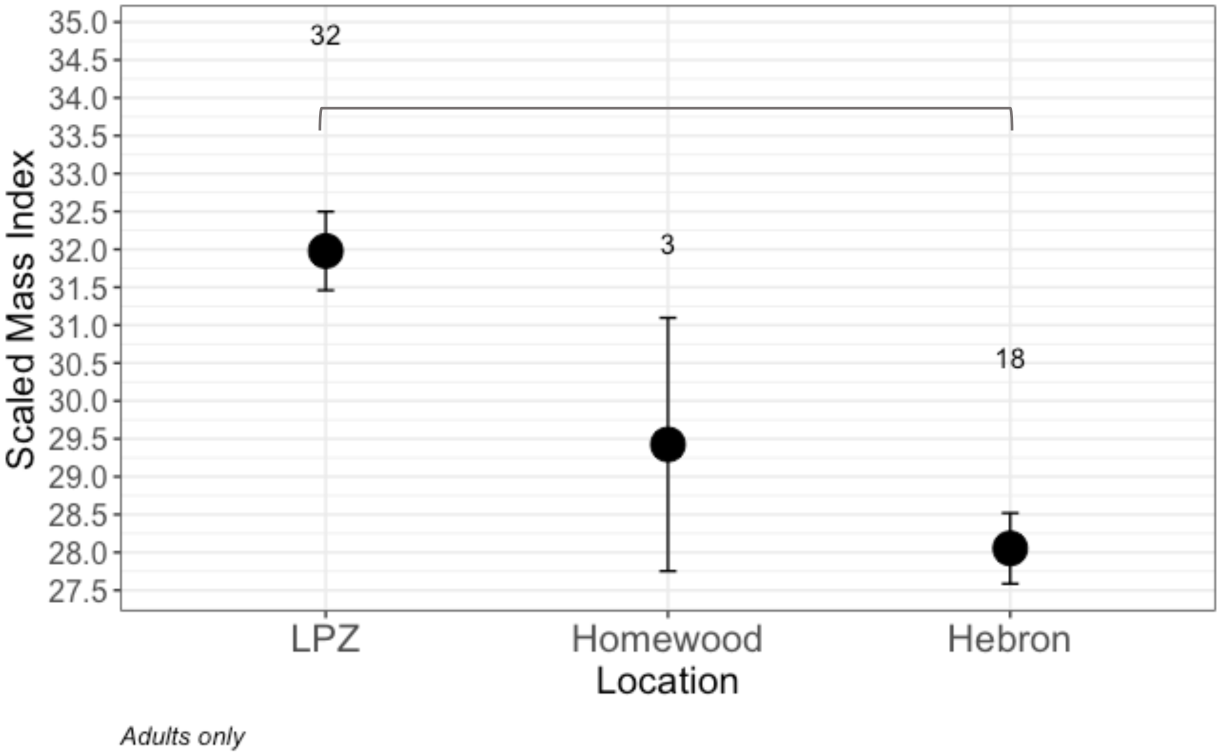


Figure 4.4 Mean (± SE) for Scaled Mass Index (SMI) of adult house sparrows by site from most urban (Lincoln Park Zoo, LPZ) to the least urban (Hebron). Numbers represent sample size. Horizontal lines indicate significant pairwise differences between sites.

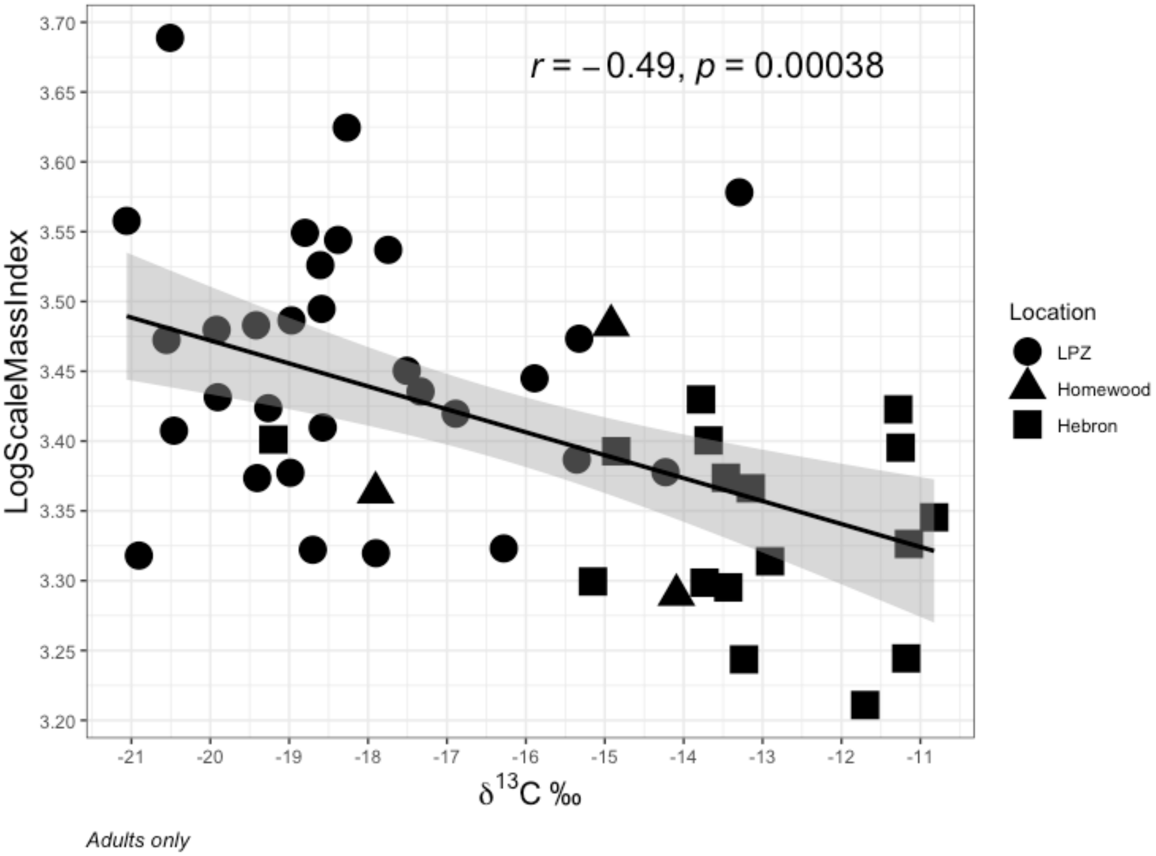


Figure 4.5 Scatter plot of adult δ^{13}C ‰ from the different sites: circles – Lincoln Park Zoo (LPZ: highly urban) n = 30; triangles – Homewood (moderately urban) n = 3; squares – Hebron (least urban) n = 17) versus the log-SMI.

Table 4.1 General linear mixed model on log transformed Scaled Mass Index. Main effects are $\delta^{13}C$ ‰ and feather corticosterone ($CORT_f$) and breeding season as the random variable. Adult house sparrows (n = 50).

Fixed effects	**Estimate**	**Standard Error**	**t-value**	**df**	**p-value**
Intercept	2.88	0.17	16.24	46	**< 0.0001**
δ13C	-0.034	0.012	-3.16	46	**0.0025**
$CORT_f$	0.11	0.069	1.60	46	0.11
δ13C:$CORT_f$	0.0076	0.0042	1.80	46	0.074

Random effect output: Breeding Season (n = 2; breeding v. non-breeding adults); variance: 2.02×10^{-20}, standard deviation: 1.42×10^{-10}; Residual: 7.30×10^{-3}, standard deviation: 8.54×10^{-2}.

4.6 Discussion

Glucocorticoids and diet signatures can be recorded in feathers. Therefore, they represent two powerful biomarkers used to understand the processes and limitations of how birds respond to their environment. We found that similar to Fairhurst et al. (2013), there was no significant relationship between $\delta^{13}C$ and corticosterone (herein $CORT_f$) indicating that diet in adult house sparrows did not influence $CORT_f$ physiology and vice versa during feather growth (in Fig. 4.7.2). Our data did not support our prediction that birds with higher $\delta^{13}C$ values (e.g., low-quality foods) would express higher integrated $CORT_f$. Our data also did not support the negative correlation found in Fairhurst et al. (2013), where Dupont's larks (*Chersophilus duponti*) with higher $\delta^{13}C$ had lower levels of $CORT_f$. The authors believed this negative correlation to be supported by the interaction between local arthropod abundance and variation in water use efficiency of plants. We did not observe a difference in adult $\delta^{15}N$ between the most urban (LPZ) and least urban locations (Hebron) (in Fig. 4.7.2). This could in part be due to similar input of nitrogen-based fertilizers for decorative plants in heavily groomed urban areas and agricultural practices associated with farms (reviewed in Harada et al. (2018)). It may support house sparrow adults feeding at the same trophic level regardless of urbanization level. Our work did focus on a single site per urban gradient, so it is imperative that samples from additional sites in different regions confirm these results. Furthermore, experimentally altering CORT levels and diet would be the most promising method to determine the absolute success of the "one sample two biomarker" technique, and we highly recommend that further research in this area.

Although the result should be interpreted with caution due to the low sample size, the most parsimonious result we obtained to support the relationship between $\delta^{13}C$ values and body condition, though surprisingly in the opposite direction of most studies (Penick, Savage, and Dunn 2015; Murray et al. 2015; Pollock et al. 2017), is that the least urbanized subjects had higher mean $\delta^{13}C$ values and lower body condition and mass than those living in the more urbanized area. This result directly contradicts that of the Shochat (2004) "credit card hypothesis", which posits that animals in more urbanized areas have lower body condition and mass when compared to those living in less urbanized areas because of predictable food availability, reduced food quality, increased predation (Dulisz, Nowakowski, and Górnik 2016; Potratz et al. 2019). However, similar to our findings, small and medium ground finches (*Geospiza fuliginosa* and *G. fortis*, respectively), which lived nearest to or among humans with access to "junk food", had the greatest body mass when compared to conspecifics living among no human activity (Knutie, Chaves, and Gotanda 2019). The patterns within body condition may very well come down to how individuals within a given population exploit resources to meet their physiological needs (Bobby Fokidis, Greiner, and Deviche 2008)

Our result contradicts the general prediction that urban animals consume more $\delta^{13}C$ rich foods associated with an anthropogenic diet; however, this could be due to the more agricultural characteristics of the least urbanized area, i.e., adult house sparrows living on or near farms have access to cornfields and corn-rich foods of domestic livestock. In fact, Gavett and Wakeley (1986b) showed that rural house sparrows had a significantly higher corn-based diet than urban birds. They concluded that this difference can be attributed to rural house sparrows having access to cornfields, corn-based domestic animal feed, and domesticated animal dung.

One potential limitation of this study is the uncertainty of how and when corticosterone is deposited into the feather (Jenni-Eiermann et al. 2015). Several studies support CORT deposition in feathers parallels modulation in the HPA axis during feather growth, with most showing that plasma CORT is positively correlated with feather CORT (Bortolotti et al. 2009; Lattin et al. 2011; Freeman and Newman 2018). However, there is some speculation for whether bloodstream corticosterone is the exclusive source for CORT deposited into keratin structures and whether CORT remains stable within the keratin matrix once deposited (Kalliokoski, Jellestad, and Murison 2019). This can ultimately change the interpretation of results from a historic record spanning months or years to that which includes only the immediate past (e.g., hours or days, see Kalliokoski, Jellestad, and Murison (2019)). Incorporation of CORT into the keratin matrix may include local production of CORT in hair follicles for mammals (reviewed in Kalliokoski, Jellestad, and Murison (2019)) and the preening oils for birds (Lattin et al. 2011), though there is insufficient data to support either. However, it is recognized that in birds, for instance, the distal portion of the feather (the oldest part) had higher CORT levels than the proximal portion, which may reflect diffusion of CORT from the skin into the feather shaft (Lattin et al. 2011).

Although our results do not directly answer what is causing declines in house sparrow populations in many areas across the globe, it does indicate that differential access to quality foods may be among the many culprits (Gadau et al. 2019). Our results highlight the positive impact urban spaces, which meet the nutritional requirements (e.g., perhaps zoos), can have in sustaining house sparrow body condition despite increased human density.

Future studies should quantify complementary indices of body condition (e.g., immune response, oxidative stress, gut microbiome, and pollution levels) and resulting fitness to better understand if the impact of urbanization on house sparrows can have both negative in some areas (e.g., sparrows in Meillère et al. (2015) in Western France) and beneficial in others as seen in our study. We encourage others to include these additional biomarkers (e.g., stress reactivity, immune response, and gut microbiota), which may extend our comprehension of how these extrinsic and intrinsic factors simultaneously affect body condition in wild animals. Key to studying these additional biomarkers is uncovering and better understanding the bidirectional and tripartite relationships among them. For instance, microorganisms can provide essential metabolic functions that can increase nutrient uptake and support immune functions (as observed in rodents and humans, (Le Chatelier et al. 2013)), however this dynamic relationship remains poorly understood in birds. Moreover, with the recent evidence for an avian microbiota-gut-brain-axis (Slevin et al. 2020), we may be able to shed light on how avian gut microbiota interacts with stress hormones and the immune system to maintain homeostasis. Uncovering these complex interactions and their mechanisms in birds will benefit our understanding of their evolutionary history and provide insights into how they are responding to environmental changes such as urbanization.

In summation, although we did not observe a difference in integrated CORT response in feathers, our work challenges the “credit card hypothesis” by demonstrating that the least urbanized subjects had lower body condition and mass than those living in the more urbanized area, which may be associated with their differential access to foods that meet their nutritional requirements. Results from this work build on the knowledge of how urbanization may affect wildlife both beneficially, as observed here, or negatively. Future work on this rapidly expanding ecosystem will be vital to understanding how wildlife are adapting to the Anthropocene.

4.7 Supplementary

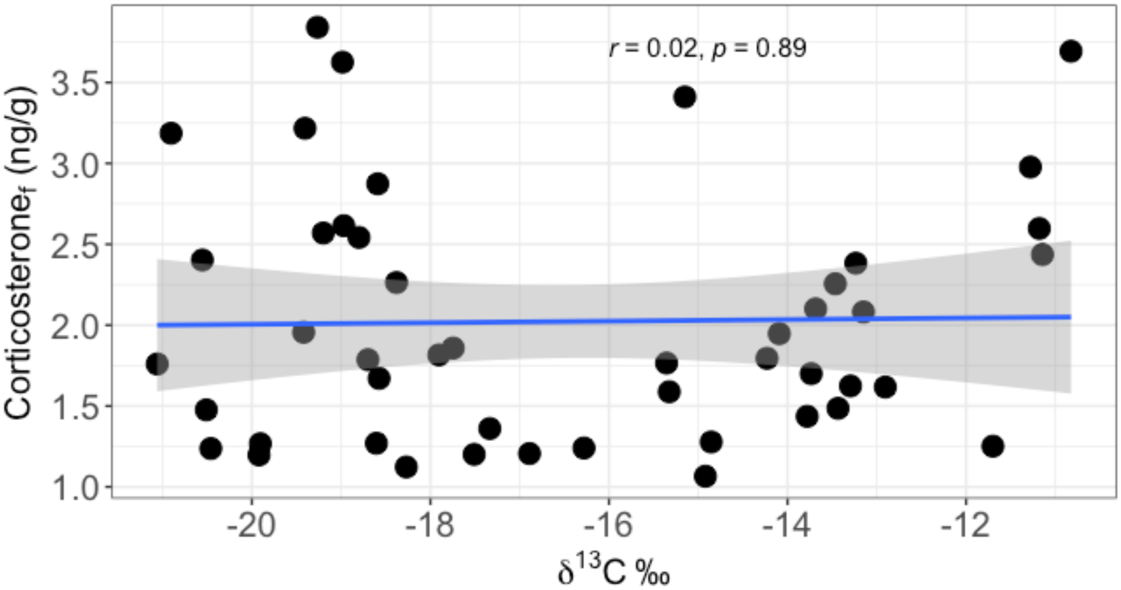


Figure 4.7.1 Pearson's correlation between corticosterone and carbon stable isotope values (n = 47 adults).

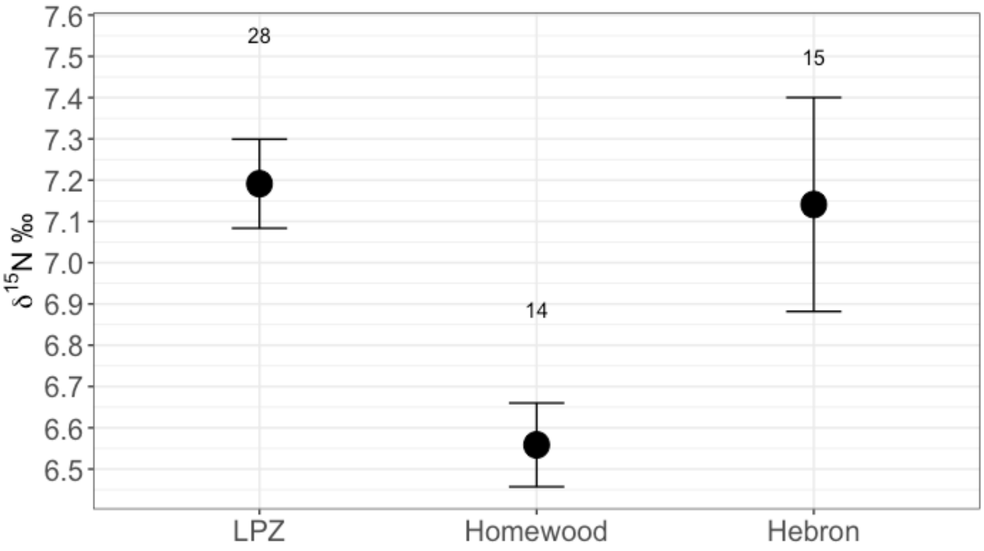
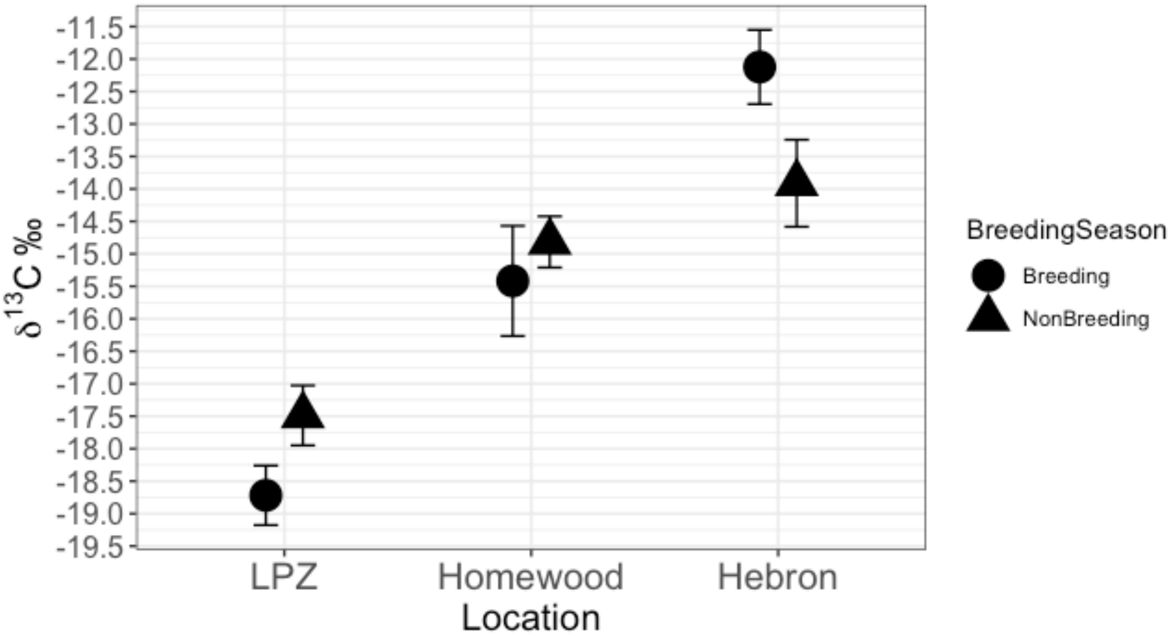


Figure 4.7.2 Nitrogen stable isotope values for adults by location. Numbers above represent sample size. Bars represent significant difference.

Table 4.7.1 Calculating difference of adult δ15N ‰ by location using post-hoc dunn test with Benjamini-Hochberg (bh) correction. Bolded p-values are significant.

Locations	p-value (adjusted by bh)	q-value
LPZ v. Homewood	**0.0020**	-3.40
LPZ v. Hebron	0.79	-0.25
Homewood v. Hebron	**0.0082**	2.77



Adults only

Figure 4.7.3 Carbon stable isotope values for adult breeding season (breeding – circle v. non-breeding - triangle) by location.

Table 4.7.2 Descriptive statistics for carbon isotopic value for adult breeding status by location. Table shows p-values calculated with pairwise Wilcoxon test and q-value statistics.

Location	Breeding Season	n	Mean Carbon	SD Carbon	SE Carbon	p-value (adjusted by fdr)	q-value
LPZ	Breeding	20	-18.71775	2.052808	0.4590218	0.11	2.89
	NonBreeding	11	17.48727	1.528204	0.4607708		
Homewood	Breeding	4	-15.41688	1.696697	0.8483485	0.73	3.08
	NonBreeding	10	-14.81598	1.245026	0.3937116		
Hebron	Breeding	7	-12.12214	1.511891	0.5714412	0.11	3.01
	NonBreeding	10	-13.91200	2.118221	0.6698404		

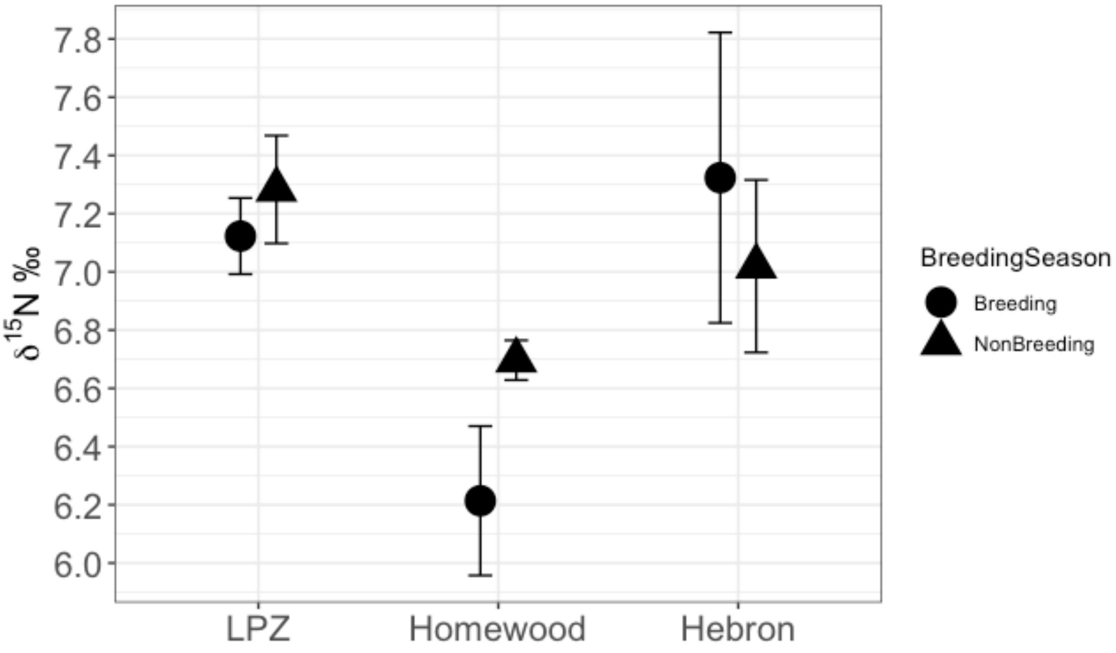


Figure 4.7.4 Nitrogen stable isotope values for adults breeding season (breeding – circle v. non-breeding – triangle) by location.

Table 4.7.3 Descriptive statistics for nitrogen isotopic value for adult breeding status by location. Table shows p-values calculated with pairwise Wilcoxon test and q-value statistics.

Location	Breeding Season	n	Mean Nitrogen	SD Nitrogen	SE Nitrogen	p-value (adjusted by fdr)	q-value
LPZ	Breeding	25	7.49	1.082	0.21	1	2.87
	NonBreeding	12	7.28	0.64	0.18		
Homewood	Breeding	20	7.43	0.99	0.22	0.12	2.89
	NonBreeding	10	6.69	0.21	0.068		
Hebron	Breeding	6	7.32	1.22	0.49	1	3.05
	NonBreeding	9	7.019	0.88	0.29		

Chapter 5

Urbanization affects baseline immune response but not glucocorticoid production nor the interaction between immune and stress responses in Midwestern house sparrows *Passer domesticus*

5.1 Abstract

Avian physiology can help individuals cope with persistent pressures associated with urbanization. Here, we measured avian innate immunity and stress hypothalamic-pituitary-adrenal (HPA) axis response as well as their interaction to assess how wild avian human-commensals are responding to the ecological challenges and opportunities along an urban gradient in midwestern, USA. We found that baseline innate immunity, measured using a bacterial killing ability assay (BKA), was positively associated with urbanization, and not the stress-induced innate immunity, which was measured after an acute stress response was induced. These results indicate that selection might exist for the rapid elimination of pathogens associated with an increased exposure to infectious diseases in urbanized areas. The HPA axis activity was determined by measuring plasma corticosterone levels (CORT). We found that CORT did not vary significantly with urbanization either at baseline or after inducing stress, and that the similarity in CORT levels along an urbanized gradient is likely unrelated to diet. Additionally, we found that the direction of the net innate response (net immunity = post-constraint BKA % – pre-constraint BKA %) was not uniform: 42% (n = 39) of birds showed a reduced immune response after constraint, 28% (n = 26) showed no change in immune response, and 30% (n = 28) showed an improvement in BKA after constraint. These results prompted us to test for a relationship to resource availability, since immunocompetence could be related to energy

allocation. For this, we quantified the seasonal average diet by evaluating the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope signatures in feathers, and then recorded weight and tarsus length. We later used these measurements as features in a principal component analysis (PCA). However, the PCA did not support variation in energy allocation as a function of innate immunity among birds with an increase, decrease, or no change in innate immunity. Our study emphasizes the importance of incorporating both intrinsic and environmental factors to interpret innate immunity and stress hypothalamic-pituitary-adrenal axis responses along an urban gradient.

5.2 Introduction

In our current era of the Anthropocene, urbanization is recognized as one of the most prevailing contributors to ecological and environmental changes. The largely irreversible transformation of both habitat structure and ecological processes in urban areas is expected to act as a set of selective pressures, favoring organismal traits that are best suited to persist or even thrive near humans (Meillère et al. 2015; Alberti et al. 2017; Isaksson 2018; Bailey 2021). Urban areas can expose organisms to greater pollution levels (e.g., light, chemicals, noise) (Riegel 1973; Bennie et al. 2014), changes to floral and faunal composition (McKinney 2006; Faeth, Bang, and Saari 2011), increased human activity (Nations 2018), reduced green space (Threlfall et al. 2016), infectious diseases (Bradley and Altizer 2007). On the other hand, urban areas can be reservoirs of advantageous features in comparison to rural areas, such as new nest site opportunities (Reynolds et al. 2019), reduced predation pressure (Møller 2012; Eötvös, Magura, and Lövei 2018) (although urban areas are associated an increase in domestic cat population (Sims et al. 2008)), and a stable source of foods (e.g., bird feeders, garbage) despite seasonal fluctuations (Chace and Walsh 2006; B.A. Evans and Gawlik 2020). Wildlife animals must thus risk extinction if they cannot either migrate or adapt to *in situ* changes through inter-generational microevolution and/or via phenotypic plasticity.

The challenges and opportunities associated with urbanized habitats have the potential to affect two important systems of physiology: (1) an efficient glucocorticoid secretion by the stress hypothalamic pituitary adrenal (HPA) axis and (2) an effective detection and elimination of microorganisms that pose a threat of infection by the innate immune system (Aristizábal and González 2013; Buchmann 2014; Riera Romo, Pérez-Martínez, and Castillo Ferrer 2016). Phenotypic divergence and the dysregulation of these two systems can have different fitness consequences for individuals from urban areas compared to rural populations. Thus, the stress HPA axis and innate immune response may help species cope with life-threating challenges by reestablishing homeostasis in the event of perturbance such as those presented with urbanization (Kouteib, Davies, and Deviche 2015; Capilla-Lasheras et al. 2017; Cummings, Hernandez, et al. 2020).

As in other vertebrates, the avian stress endocrine and innate immune systems are networks of cells, tissues, and biomediators (Huber et al. 2017). Although historically studied as two separate systems, it is increasingly evident that the endocrine and immune systems can work synergistically to protect against threats to homeostasis (Dhabhar 2014). The crosstalk that is fundamental to coregulating homeostasis, which plays a strong influence on the adaptation and/or coping strategies of wildlife, is based on the two systems sharing (1) biomediators (e.g., neurotransmitters, hormones) and (2) receptors (e.g., G-protein coupled receptor) (Ortega et al. 2021). Studies have shown that circulating plasma corticosterone (the primary glucocorticoid in Aves; herein referred to as CORT) changes frequently to meet a suite of seasonal to daily demands such as finding mates (Silverin 1998) and reallocating energy after fleeing or fighting antagonists (Scheuerlein, Van't Hof, and Gwinner 2001). In fact, an acute stress response – namely the short-term production of CORT with an efficient negative feedback – is considered adaptive because it promotes gluconeogenesis to meet the energy demands for reproduction and survival (Exton 1972; Dallman et al. 1993; Lattin and Romero 2015). An acute increase in CORT can also have some immunoenhancing effects on the immune response, helping organisms recover from potential challenges (e.g., wounds or infection) for which activation of the stress HPA axis acts as an early warning signal (Dhabhar and Mcewen 1997; Dhabhar and McEwen 1999; Dhabhar 2014). However, some studies have shown that an immediate increase in CORT can have an opposite (i.e., immunosuppressant) influence on the immune response (El-Lethey, Huber-Eicher, and Jungi 2003; Yang et al. 2015; Gao, Sanchez, and Deviche 2017). It is also widely accepted that a persistent production of glucocorticoids can reduce efficacy of the overall immune response including the innate arm, thus increasing susceptibility to new infections or causing the exaggeration of others (reviewed in Marketon and Glaser (2008)). The immunosuppressant effect of stress in such cases is believed to redirect resources toward activities that are immediately valuable for survival, such as maintaining elevated glucocorticoids when an enhanced immune response might be too costly and therefore incompatible with an individual’s fitness (Sapolsky, Romero, and Munck 2000; Martin et al. 2012).

A spectrum of physiological interactions that allow animals to respond effectively to an urban world have been demonstrated for many wildlife species (A.A. Cohen et al. 2012). For example, both baseline and stress-induced CORT levels were significantly lower in urban tree lizards *Urosaurus ornatus* compared to their rural counterparts, suggesting dampened HPA activity in urban individuals as a result of chronic stress (or perhaps access to increased food resources) (French, Fokidis, and Moore 2008). Attenuation of the stress HPA axis to become less sensitive to chronic urban stressors is believed to help wildlife avoid the negative effects of elevated levels of circulating glucocorticoids (Atwell et al. 2012). (French, Fokidis, and Moore 2008) also found an increase in the ratio of heterophils to lymphocytes (H: L) that was consistent with population differences in baseline CORT levels, and they found that urban lizards also had higher leukocyte count. However, when comparing the immune response of four amphibians, results showed no differences between urban and rural populations (Iglesias-Carrasco, Martín, and Cabido 2017), and Bókony et al. (2021) found that baseline and stress-induced CORT levels were higher in urban common toad *Bufo bufo* tadpoles than in those living in natural areas. Different ecological and environmental challenges or opportunities associated with urban areas may ultimately have both direct and indirect effects on the stress and immune responses, leading to variation in physiological responses between urban animals and their rural counterparts.

Birds are considered valuable indicator species for monitoring the health of environments, including both overall biodiversity and levels of chemical pollution (Burger and Gochfeld 2004; Gregory 2006). They have also been shown to have varying physiological responses to urbanization, but some studies find opposing results or find no difference in stress and immune responses between conspecifics from urban and rural populations (Carbó-Ramírez and Zuria 2017; Injaian et al. 2020; Ibáñez-Álamo et al. 2020), respectively). For example, in respect to the innate immune function, Bailly and colleagues (2016) showed that, across two years, great tit nestlings *Parus major* in urban areas had significantly less haptoglobin (Hp), a biomarker for inflammatory response, when compared to nestlings in forested areas. Conversely, when compared to rural individuals, urban curve-billed thrashers *Toxostoma curvirostre* and northern mockingbird *Mimus polyglottos* had higher heterophil to lymphocyte ratio (H/L) and higher leukocyte count, which suggested chronic stress and infection (Bobby Fokidis, Greiner, and Deviche 2008). In respect to the HPA stress response, studies have found higher CORT levels in urban individuals of common blackbirds *Turdus merula* (Meillère et al. 2016), house sparrows *Passer domesticus* (Beaugeard et al. 2019), and salamanders *Eurycea tonkawae* (Meillère et al. 2016; Gabor et al. 2018; Beaugeard et al. 2019) than in their rural conspecifics, but Crino et al. (2013) showed the opposite effect of urbanization on avian physiology. These researchers found that experimentally elevated traffic noise, a proxy for anthropogenic noise, was associated with significantly lower CORT levels coupled with a dampened CORT response when compared to the control group.

Perhaps unsurprisingly given the young age of this field (reviewed in Isaksson et al. (2020)), there is still no consensus for how urbanization affects wildlife physiology including that of the stress HPA and innate immune responses in birds. The heterogeneous results of these physiological responses thus make it difficult to generalize about the impact that urbanization has on wildlife animals. One possible explanation for this heterogenicity is that so few studies (e.g., (Chávez-Zichinelli et al. 2010), Stress responses of the House Sparrow (Passer domesticus) to different urban land uses; (Cummings, Hernandez, et al. 2020; Ibáñez-Álamo et al. 2020). Cummings, Hernandez, et al. (2020) have examined HPA stress and innate immune responses as interacting systems. Instead, most studies perceived stress and immune response as independent systems governing species resilience and sensitivity to an increasingly urbanizing world.

Here, we measured the effects of varying degrees of urbanization on the stress HPA axis and innate immune responses both independently and in combination, using the house sparrow (*Passer domestics*) as our test animal. Furthermore, because energy and nutrient quality can act as limiting factors in stress (Astheimer, Buttemer, and Wingfield 1992; Kitaysky et al. 2001; Kitaysky, Wingfield, and Piatt 2001) and immune response (Strandin, Babayan, and Forbes 2018), we quantified diet, using stable isotopes carbon and nitrogen. Our objectives were: (1) examine whether circulating CORT levels influence the innate immune response; (2) determine whether house sparrow stress response varied along an urban gradient, i.e., baseline CORT (pre-constraint), stress-induced CORT (post-constraint), and magnitude of CORT response (post-constraint CORT levels – pre-constraint CORT levels); (3), much like the second objective, determine whether house sparrow innate immune response differed along an urban gradient; and (4) test whether the interaction between stress and the innate immune response varied along an urbanized gradient. We hypothesized that the effect of urbanization on CORT and immune response is related to how the two systems interact with each other. Such an interaction would suggest that there are multiple physiological interactions that aid wildlife species in coping with urbanization. For example, immune response could be low within a population owing to the immunosuppressant effect of elevated CORT and not because there are no infectious challenges. Research in this area can highlight why studies within the same species show conflicting results.

5.3 Materials and Methods

5.3.1 House sparrow collection

House sparrows (n = 149) were captured from nine sites along an urbanized gradient in the midwestern (Illinois and Indiana) United States during autumn (August – December) 2019 and 2020. We captured birds between 6 am and 11 am. A gradient from least to most urban was calculated using a principal component analysis of the following features: canopy cover, impervious surface, and human density within a 2 km buffer of each site (the home range of house sparrows (P.E. Lowther and Cink 1992)). We used a combination of mist nests and Tomahawk live-traps baited with sunflower seeds to collect juveniles (n = 76) and adults (n = 73). Upon capture, we recorded age and sex, and collected body feathers (n = ~25 feathers) from the left breast of each individual bird (for diet analysis). Age and sex were determined based on plumage color. Individuals with black beaks and black bibs were considered adult males, birds with light gray beaks and black bibs were considered juvenile males, and individuals with dark gray beaks and noticeably striped backs were tallied as adult females. Molecular sexing was conducted for individuals whose sex and age were ambiguous. Each house sparrow was then fitted with a U.S. Geological Survey aluminum leg band (permit# 09924). Feathers were stored in opaque envelopes at room temperature. All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee (ACUP # 72510).

Figure 5.1 (a) Location of nine study sites across Illinois (n = 7) and Indiana (n = 2). The two overlapping sites in Evanston are highlighted in the orange rectangle. (b) Visualization from least urban (Dunes, IN) to most urban (Pilsen, IL). Arial images from Google Earth Pro; Version 7.3.4.8248 (64-bit).

5.3.2 Physiological measurements

To assess stress HPA axis function and innate immune response, we took blood samples from the brachial vein of captured birds at two time points using techniques recommended by The Ornithological Council (Fair and Jones 2010). Baseline (pre-constraint) blood was collected within the first 5 mins of capture (L.M. Romero and Reed 2005; Bonier et al. 2009; Injaian et al. 2020). Acute stress-induced (post-constraint) blood was collected after 30 mins of bird constraint in opaque cloth bags, similar to Gao et al. (2017). We swabbed the skin around the brachial vein with 70% ethanol and then pierced the vein using a sterile 25-gauge needle. Blood was collected into sterile heparinized microcapillary tubes, which were placed on ice. On return from the field, samples were centrifuged for 10 minutes at 10,000 rpm. The plasma was then removed, divided into two aliquots (one for the BKA assay and one for the CORT assay), and frozen at −70°C until assay.

5.3.3 Urban score

We collected house sparrows across nine sites in the Midwestern, USA (Fig. 5.1). The magnitude of urbanization was calculated using a principal component analysis (R package, stats::prcomp) that included three variables: percent canopy cover, percent impervious surface, and human density within a 2 km buffer zone (Table 5.1; see supplementary 5.1 for R code). A single 2 km buffer zone is the estimated home range of house sparrows (P.E. Lowther and Cink 1992). Two of the nine collection sites were less than 2 km apart in Evanston, IL (~0.96 km apart), permitting some possible overlap in house sparrow home range. We thus calculated a single average PC1 value to represent these two sites.

Table 5.1 Principal Components Analysis using variables canopy cover (%), impervious surfaces (%), and human density (number of humans) within a 2km buffer to calculate the resulting urban score. Features were centered and scaled. Urban scores are PC1 values, which explained ~77.5% of the variance among variables. Positive values represent higher urban scores at sites. Negative values represent lower urban scores at sites.

Sources: canopy cover: NLCD 2016 USFS Tree Canopy Cover; impervious surfaces: NLCD 2016 Percent Developed Imperviousness ; human density: SILVIS LAB, Spatial Analysis for Conservation and Sustainability. Refer to R script (urban_score_pca) for additional information.

Site	Canopy Cover (%)	Impervious Surfaces (%)	Human Density	Urban Score
Pilsen, IL	0.75	77.18	3750.56	1.57
Lincoln Park, IL	1.45	47.78	6876.68	1.283
Evanston, IL	5.04	52.82	4390.68	0.718
Hyde Park, IL	3.12	44.37	4199.94	0.492
Pullman, IL	3.19	43.63	1373.98	-0.169
Hammond, IN	2.72	45.19	714.36	-0.244
Homewood, IL	12.01	36.46	1242.84	-0.853
Dunes, IN	41.03	5.60	31.43	-3.514

5.3.4 Bacteria killing ability (BKA) assay for innate immunity

To quantify bacteria killing ability, a broad-spectrum measure of innate immunity, we followed the procedure developed by French and Neuman-Lee (2012), calibrated it for house sparrow. We plated10 µL of sample that had been diluted 1:4 in phosphate-buffered saline (PBS), in duplicate. We also plated a positive control (10 μL of PBS) and negative control (12 μL of PBS) in duplicate. A serial dilution of cow plasma was also included as an inter-assay control; these Cow controls were plated at 3 dilutions, from 0.03125 to 0.007813, plated in duplicate, with each plate having a final volume of 18 μL.

Two μL of 10^4 bacteria ml^{-1} *Escherichia coli* (ATCC #8739) was added to each sample, to the positive controls, and to the cow controls. *E. coli,* a Gram-negative and potentially intestinal pathogen, is the most common bacterium used in eco-immunology studies due to its ecologically relevance (Irene Tieleman et al. 2005; Matson, Tieleman, and Klasing 2006; Demas et al. 2011). Each plate was vortexed for 1 min. at 500 rpm and then incubated for 30 min at 37 ºC. We then added 125 μL of tryptic soy broth to each well, vortexed the plate for 1 min. at 300 rpm, and measured for absorbance at 300 μm on a spectrophotometer. The plate was incubated for 12 hours at 37 ºC and read again at 300 μm on a spectrophotometer. This first reading served as an internal control for the later growth reading. Bacteria killing ability was calculated as

$$\left(1-\frac{(sample\ end - sample\ baseline)}{(control\ start - control\ end)}\right) * 100\%.$$

Intra-sample variation was <5.4% and inter-assay variation was 9.7%

5.3.5 Plasma corticosterone hormone assay for stress response

Plasma CORT concentrations were quantified using a commercially available EIA kit (Arbor Assays DetectX Corticosterone EIA Kit, Product # K014). The detection limits of the CORT assay were 78 pg/mL to 10,000 pg/mL. Samples were diluted 1:10 with assay buffer and run according to kit directions. All samples were assayed in duplicate. In total, eight CORT assays were performed. Mean intra-assay variation of duplicate samples was 5.6 ± 0.4%. Mean inter-assay variation of two quality control pools was 10.6 ± 2.0%.

5.3.6 Stable isotopes for diet

Stable isotopes carbon and nitrogen can reflect the diet of animals (Thompson and Furness 1995). Briefly, the carbon isotopic signature of the animal reflects the metabolic differences between photosynthesis pathways in plant food sources (e.g., C3 versus C4 plants): a three-carbon compound is associated with C3 plants (e.g., cereal grains, sunflower) while a four-carbon compound is associated with C4 plants (e.g., corn, sugarcane). Diets enriched in C4 plants will have enriched $\delta^{13}C$ compared to diets dominated by C3 plants. Similarly, nitrogen is enriched with trophic level, wherein carnivores have higher $\delta^{15}N$ signatures than omnivores and omnivores have higher $\delta^{15}N$ signatures than herbivores.

To measure these signatures, pulverized feather samples were weighed to a target sample size of 0.5mg ± 0.005mg on a micro-analytical balance (Mettler Toledo, Columbus, Ohio) and placed into 3.5 x 5 mm tin capsules (Costech Analytical Technologies INC, Valencia, CA) in duplicates. The stable isotope compositions ($\delta^{15}N$ and $\delta^{13}C$) of samples were determined in the Stable Isotope Biogeochemistry Laboratory of the Department of Earth and Planetary Sciences, Northwestern University (Chicago, Illinois), using continuous-flow mass spectrometry. The system consisted of an Elemental Combustion System 4010 (Costech Analytical Technologies INC, Valencia, CA) with a ZeroBlank autosampler (Costech Analytical Technologies INC, Valencia, CA), connected to a Thermo Delta V Isotope Ratio Mass Spectrometer via a Conflo IV (Thermo Fisher Scientific INC, Waltham, MA), controlled by Isodat software (Thermo Fisher Scientific INC, Waltham, MA). The reported $\delta^{15}N$ and $\delta^{13}C$ values were corrected using in-house working standards, calibrated to acetanilide and urea standards supplied by Arndt Schimmelmann of Indiana University (Schimmelmann et al. 2009). Stable isotope results had a typical precision, with a standard deviation of 0.3‰ for $\delta^{15}N$, and 0.2‰ for $\delta^{13}C$.

5.3.7 Statistical analysis

All analyses were performed in R (v4.0.3; R Core Team, 2020). During hypothesis testing, we fitted linear mixed models with maximum likelihood to test whether bacteria killing ability (%), CORT levels (ng/mL), and the interaction between BKA/CORT differed along an urbanized gradient. We also tested whether CORT influences BKA. Each response variable was separated by baseline, stress-induced, and net values (stress-induced minus baseline). For BKA

(%), models were fitted with CORT, bird age (two level factor: adult and juvenile), carbon isotopic value, nitrogen isotopic value, and urban score, all as fixed variables. For CORT, models were fitted with bird age, carbon, nitrogen, and urban score as fixed effects. Similarly, for the interaction (BKA divided by CORT to obtain a single value), models were fitted with age, nitrogen, carbon, and urban score as the fixed effects. For all models, we used bird sex as a random variable: we had three factors, -- male, female, and unknown, i.e., 11 juveniles -- and so did not want further reductions to the analytical sample size. Sample sizes vary between tests because of sampling limitations (e.g., insufficient volume of blood for both BKA and CORT analyses). We also included location nested into collection year as a random variable to account for samples collected in Fall 2019 and Fall 2020.

Following a frequentist approach as used by Bókony et al. (2012) and Salleh Hudin et al. (2018), we performed all model selection using a stepwise approach, starting with a full model that included all independent variables. We removed non-significant factors one at a time until we reached the most parsimonious model. Statistics and p-values ($p < 0.05$) reported in tables refer to the final models (i.e., only significant terms are included). Statistics and p-values of non-significant terms were obtained by fitting each non-significant term separately into the minimal model (see Table 5.7.1). We did not run any interactions for these models due to fear of over-fitting with too many terms, given the limited sample sizes (reported in results). We first tested for data normality using the Shapiro-Wilk test. Data transformation did not improve normality for those failing this test, and so untransformed values are used throughout. For data where the Shapiro-Wilk test was $p < 0.05$ (not normal), we used a non-parametric to the Kruskal-Wallis test to determine statistical difference between groups (e.g., CORT levels between juveniles and adults). For normally distributed data, we used a student's t-test to determine statistical difference between groups (e.g., CORT levels at baseline v. stress-induced). We used Pearson's correlation to determine the strength and direction of significantly identified relationships from the LMMs above. All final models were checked for assumptions of equal variance and normality of residuals using a qqplot and Levene's test, respectively. Residuals that disobeyed the assumptions are reported but should be considered under those limitations.

Because birds (n = 93) exhibited both positive and negative immune responses as well as no significant response, we assessed the contributions to that variation from different factors, i.e., related to diet ($\delta^{15}N$, $\delta^{13}C$) and body condition (weight (g) and tarsus length (mm)). We focused on diet and body condition due to their causative relationship to immune function as they represent access to and quantity of nutrients required for an effective immune response (Wilcoxen et al. 2015; Hwang et al. 2018; A.D. Smith et al. 2018; Strandin, Babayan, and Forbes 2018; Ruhs, Martin, and Downs 2020). We separately quantified the proportion of individuals within a given net immune response direction as a measure of immunocompetence. A principal component analysis was run with $\delta^{15}N$ and $\delta^{13}C$ as separate proxies for diet, and with body weight (g) and tarsus length (mm) as proxies for body condition. The direction of net immune response as expressed using the first two axes (PC1 and PC2) was plotted with an ellipse capturing 95% variability to highlight any distinctions among the three immunocompetence groups.

5.4 Results

We captured a total of 149 house sparrows (n = 76 juveniles and n = 73 adults). Plasma was sufficient to quantify net CORT for 117 birds, to quantify net BKA % for 93 birds, and to quantity both net-CORT and net-BKA % for 76 birds.

5.4.1 Variation in corticosterone

We found a significant increase in CORT levels after 30-min of restraint in cloth bags (paired t-test: $p < 0.001$; df = 116; t = -18.42). Baseline CORT was significantly higher in adults (17.40 ± 9.23 ng/mL, n = 61) when compared to juveniles (13.75 ± 7.07 ng/mL, n = 57) (Kruskal-Wallis $\chi^2 = 4.91$, df = 1, p-value = 0.027; Fig. 5.2). Stress-induced and net- CORT were positively correlated with $\delta^{13}C$ (i.e., the C4-plant indicator; stress-induced CORT: r = 21, p = 0.024, n = 117, Fig. 5.3; net CORT: r = 0.2, p = 0.03, n = 116, Fig. 5.4). However, when separated by age, we found one significant correlation, which was a positive correlation between net CORT and $\delta^{13}C$ (r = 0.4, p = 0.0086, n = 57; Fig. 5.4) for juveniles. Although the model (Table 5.2) suggested age as a predictor of net CORT, we surprisingly found that although juveniles (25.27 ± 12.80 ng/mL, n = 57) had higher net CORT than adults (22.19 ± 15.08 ng/mL, n = 60), the difference was not statistically significant (Kruskal-Wallis: $\chi2 = 2.7663$, df = 1, p = 0.096; Fig. 5.5).

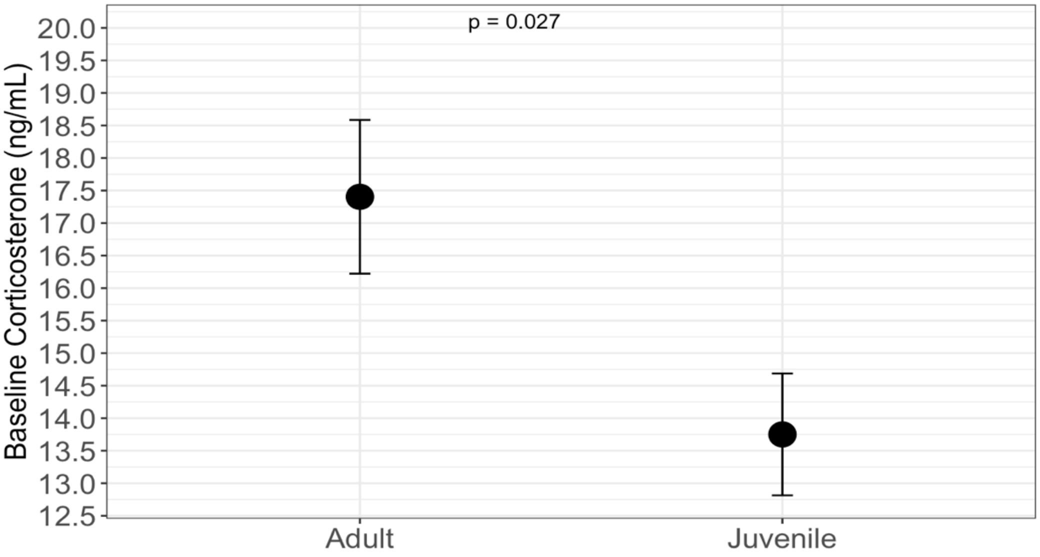


Figure 5.2 Mean and standard error of baseline corticosterone for adults (17.40 ± 1.18 ng/mL, n = 61) and juveniles (13.75 ± 0.93 ng/mL, n = 57). $p < 0.05$ marks significance.

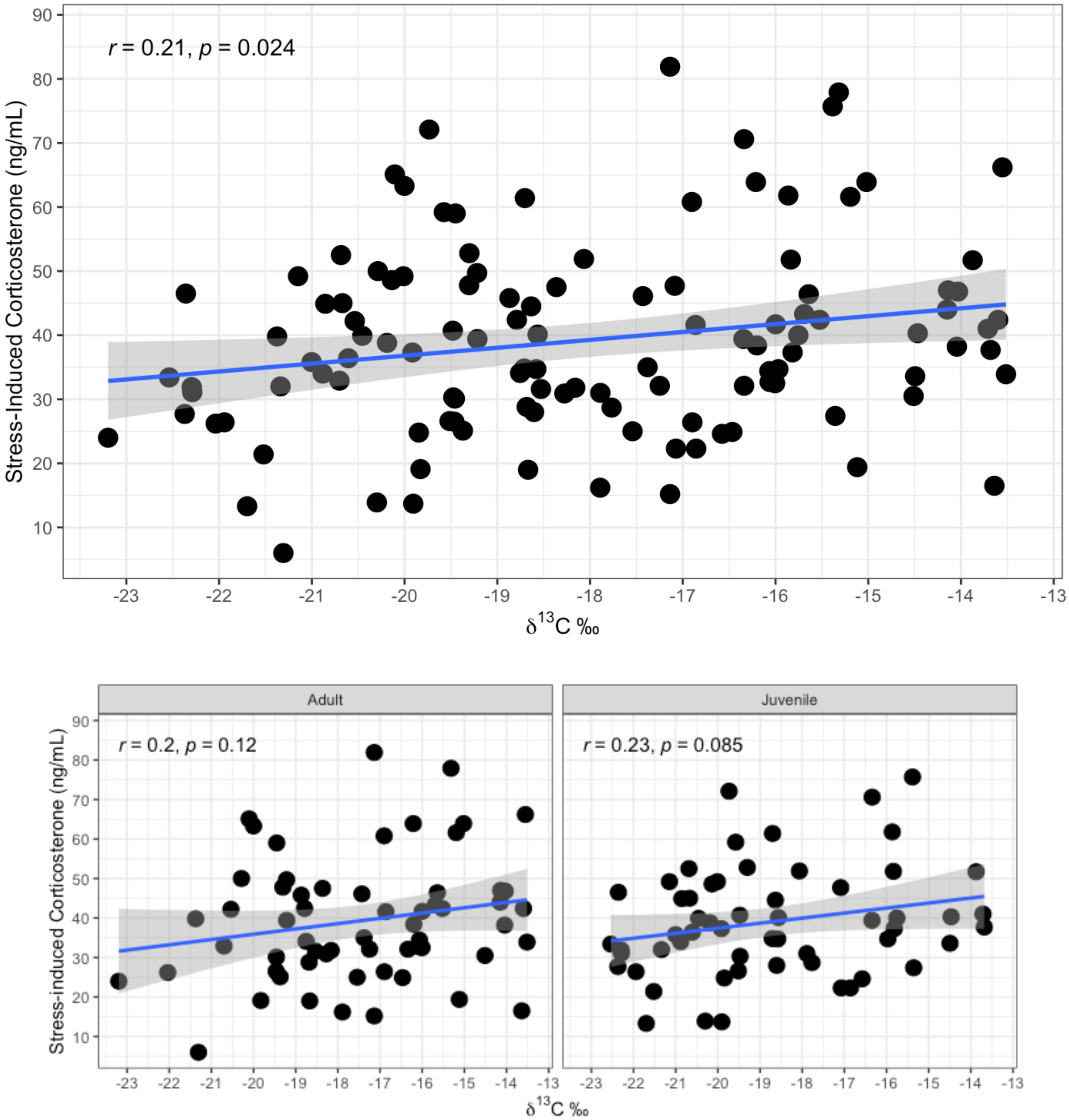


Figure 5.3 (top) Pearson's correlation (r = 0.21, p = 0.024, n = 117) for the relationship between δ^{13}C ‰ and stress-induced CORT (ng/mL) for both adults and juveniles. Pearson's correlation for adults δ^{13}C ‰ and stress-induced CORT (lower left; r = 0.2, p = 0.12, n = 60), and for juveniles (lower right; r = 0.23, p = 0.085, n = 57)

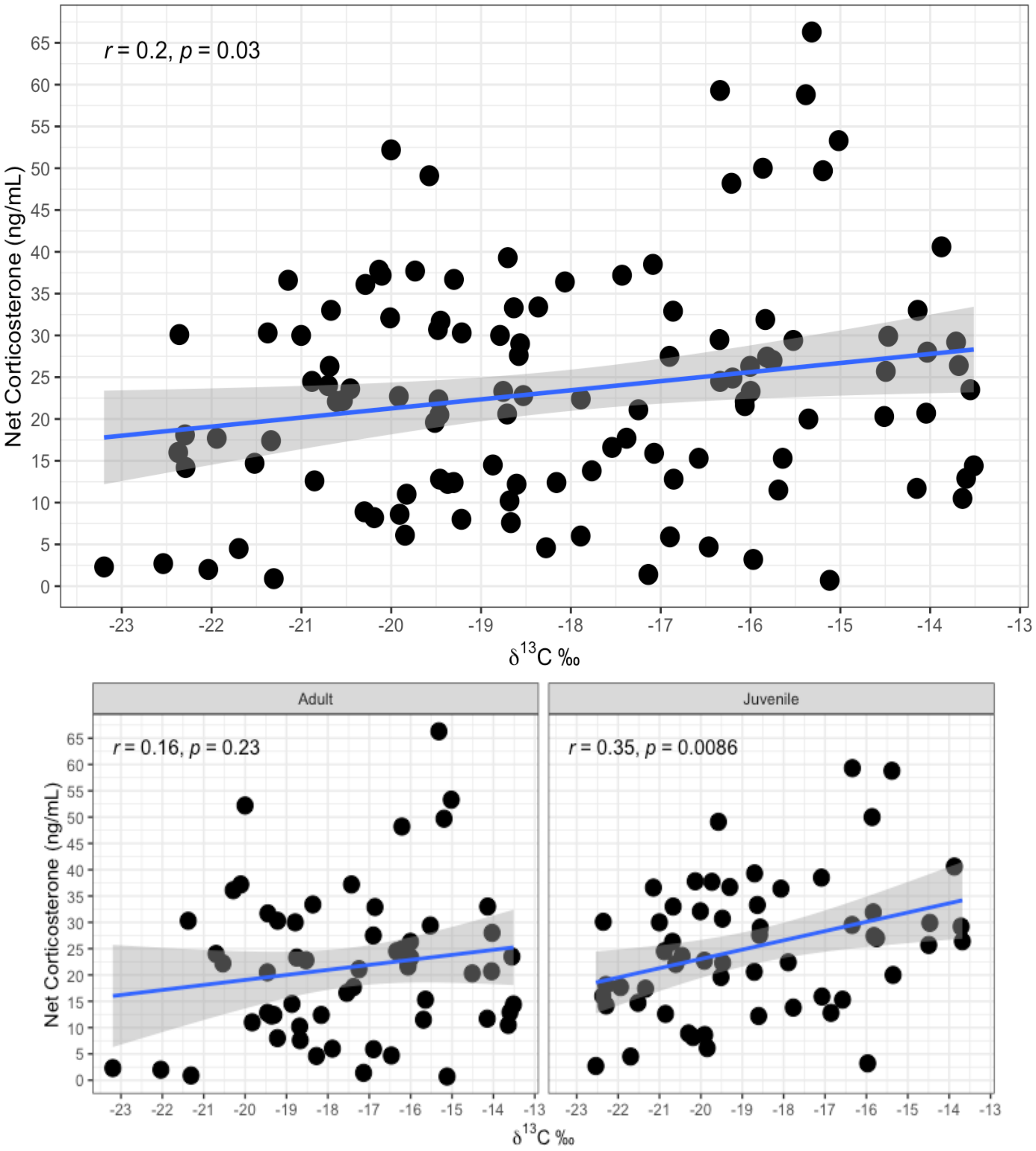


Figure 5.4 (top) Pearson's correlation (r = 0.2, p = 0.03, n = 116) for the relationship between δ^{13}C ‰ and net CORT (ng/mL). Pearson's correlation for adults δ^{13}C ‰ and net CORT (lower left; r = 0.16 p = 0.23, n = 59), and for juveniles (lower right; r = 0.4, p = 0.0086, n = 57).

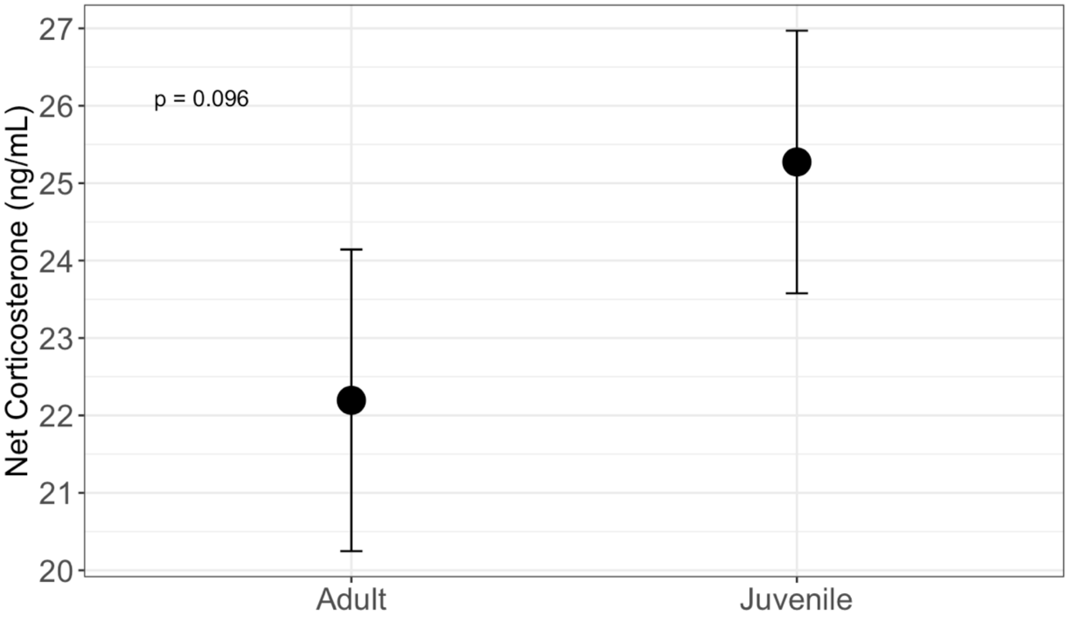


Figure 5.5 Mean and standard error of net corticosterone for adults (22.19 ± 1.94 ng/mL, n = 60) and juveniles (25.27 ± 1.69 ng/mL, n = 57).

5.4.2 Variation in Bacteria Killing Ability

Baseline BKA % increased significantly along an urbanized gradient, both when adults and juveniles were analyzed together (r = .32, p = 0.00087, n = 106; Fig. 5.6) and when they were analyzed separately (adults: r = 0.31, p = 0.029, n = 50; juveniles: r = 0.36, p = 0.0062, n = 56; Fig. 5.6).

The only significant relationship between BKA and CORT identified by the model was that of stress-induced blood (Table 5.2). Upon further inspection, we found a positive correlation between stress-induced BKA and stress-induced CORT within juveniles (r = 0.3, p = 0.051, n = 42; Fig. 5.7), but did not find a significant relationship within adults (stress-induced: r = 0.057, p = 0.73, n = 40) or when juveniles and adults were grouped together (r = 0.17, p = 0.12, n = 82). There was also no significant relationship between CORT and BKA % either at baseline or under stress-induced conditions when adults and juveniles were grouped or when analyzed separately (p-values all > 0.05).

Our mixed model suggested that stress-induced BKA was negatively associated with $\delta^{13}C$ (Fig. 5.7.2). However, Pearson's correlation showed no significant relationship when adults and juveniles were grouped or when analyzed separate (Fig. 5.7.1; grouped: r = -0.15, p = 0.15, n = 97; adults: r = -0.21, p = 0.16, n = 45; juveniles: r = -0.055, p = 0.7, n = 52).

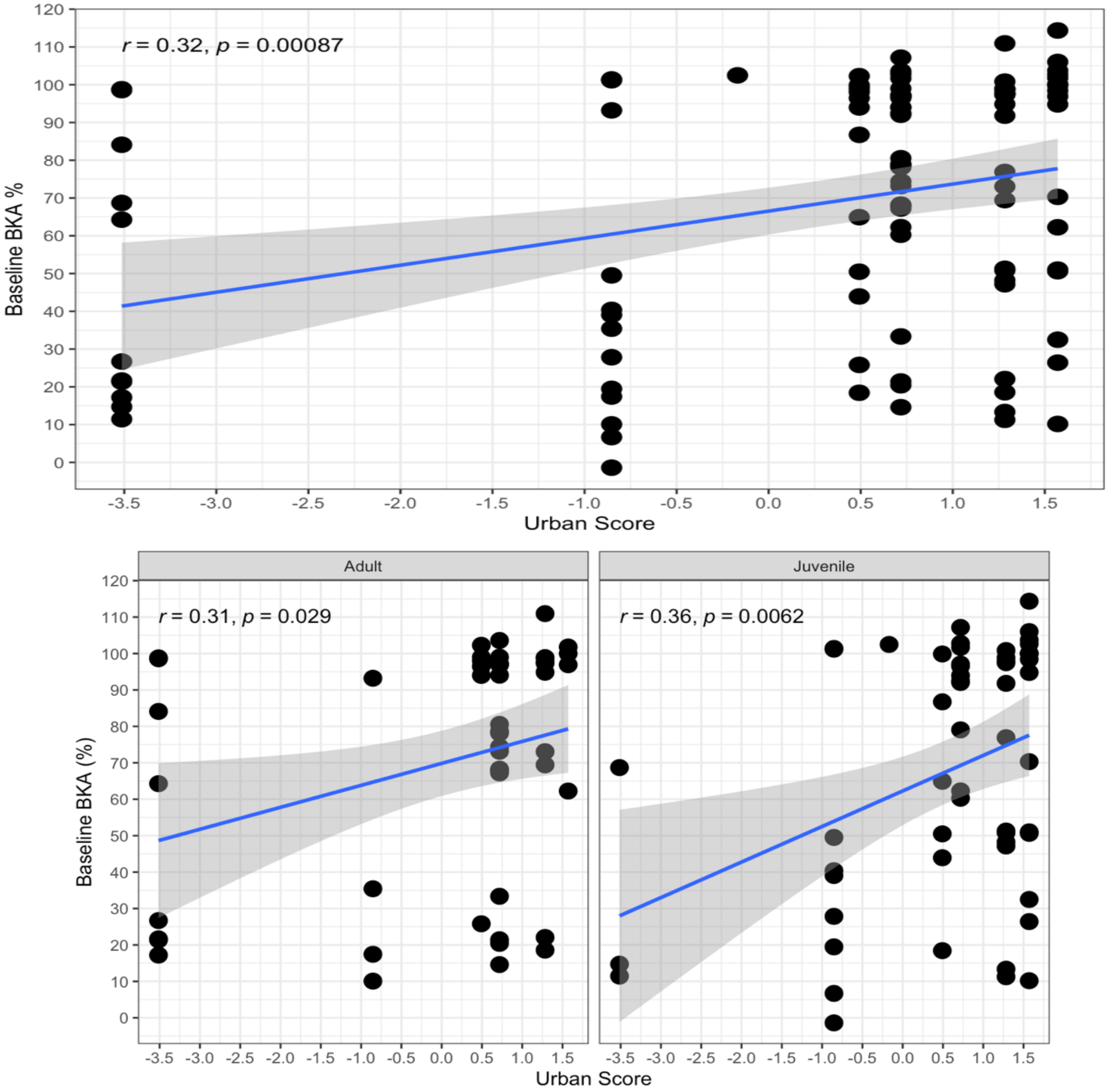


Figure 5.6 Urban score (logged) in relation to baseline bacteria killing ability (%). Top panel shows adults and juveniles in the same plot with Pearson's correlation calculated (n = 106, r = 0.32, p = 0.00087). Bottom panels show adults (left, r = 0.31, p = 0.029, n = 50) and juveniles (right, r = 0.36, p = 0.0062, n = 56) for Pearson's correlation. Standard error with 95% confidence interval shaded in gray.

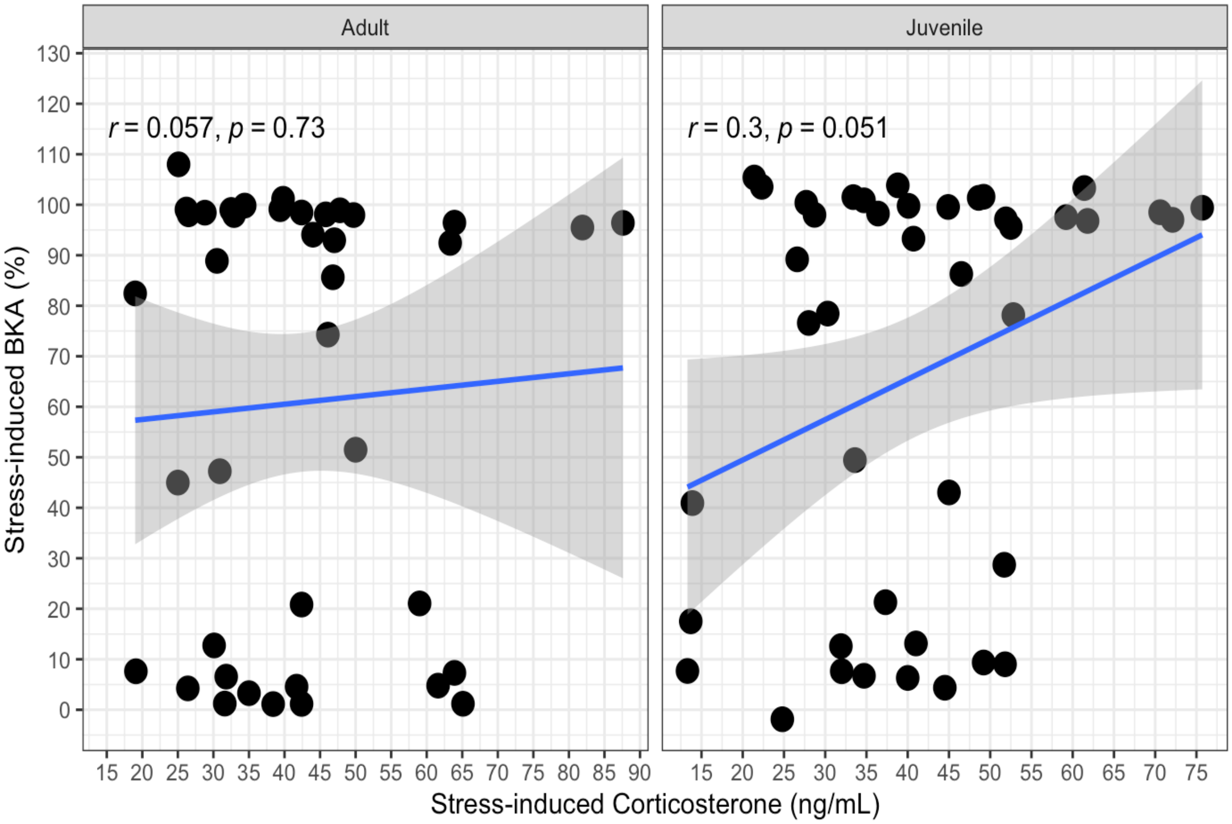


Figure 5.7 Pearson's correlation stress-induced corticosterone (ng/mL) in relation to stress-induced bacteria killing ability (%) for adults (r = 0.057, p = 0.73, n = 40) and juveniles (r = 0.3, p = 0.051, n = 42). Standard error with 95% confidence interval shaded in gray.

5.4.3 Interaction between corticosterone and immune response along an urbanized gradient

A significant negative correlation (r = -0.27, p = 0.014, n= 82) exists between the stress-induced interaction $\frac{BKA\%}{CORT^{ng}/_{mL}}$ and δ^{13}C as a measure of environment. The relationship was no longer significant when individuals were separated by age (p > 0.05) (Fig. 5.8).

Table 5.2 Linear mixed model by maximum likelihood explaining variation in baseline-, stress-induced-, and net- bacteria killing ability (BKA %) and corticosterone (CORT ng/mL), and the interaction BKA / CORT $\frac{BKA\%}{CORT^{ng}/_{mL}}$. Models selected by using a stepwise approach starting from full models and removing predictor variables with P > 0.05.

Dependent Variable	Predictor Variable	Estimate	Std. Error	df	t-value	p-value
baseline CORT	age	-3.37	1.49	116.91	-2.26	0.025
stress-induced CORT	δ^{13}C ‰	1.88	0.57	99.32	3.28	0.0014
net CORT	δ^{13}C ‰	1.92	0.52	93.11	3.65	0.00042
	age	7.58	2.50	108.10	3.025	0.0031
baseline BKA	urban score	6.38	2.22	31.44	2.87	0.0072
stress-induced BKA	δ^{13}C ‰	-4.39	1.91	81	-2.29	0.024
	stress-induced CORT	0.60	0.30	81	1.97	0.051
net BKA	-	-	-	-	-	-
baseline $\frac{BKA\%}{CORT^{ng}/_{mL}}$	-	-	-	-	-	-
stress-induced $\frac{BKA\%}{CORT^{ng}/_{mL}}$	δ^{13}C ‰	-0.13	0.058	77.60	-2.22	0.02
net $\frac{BKA\%}{CORT^{ng}/_{mL}}$	-	-	-	-	-	-

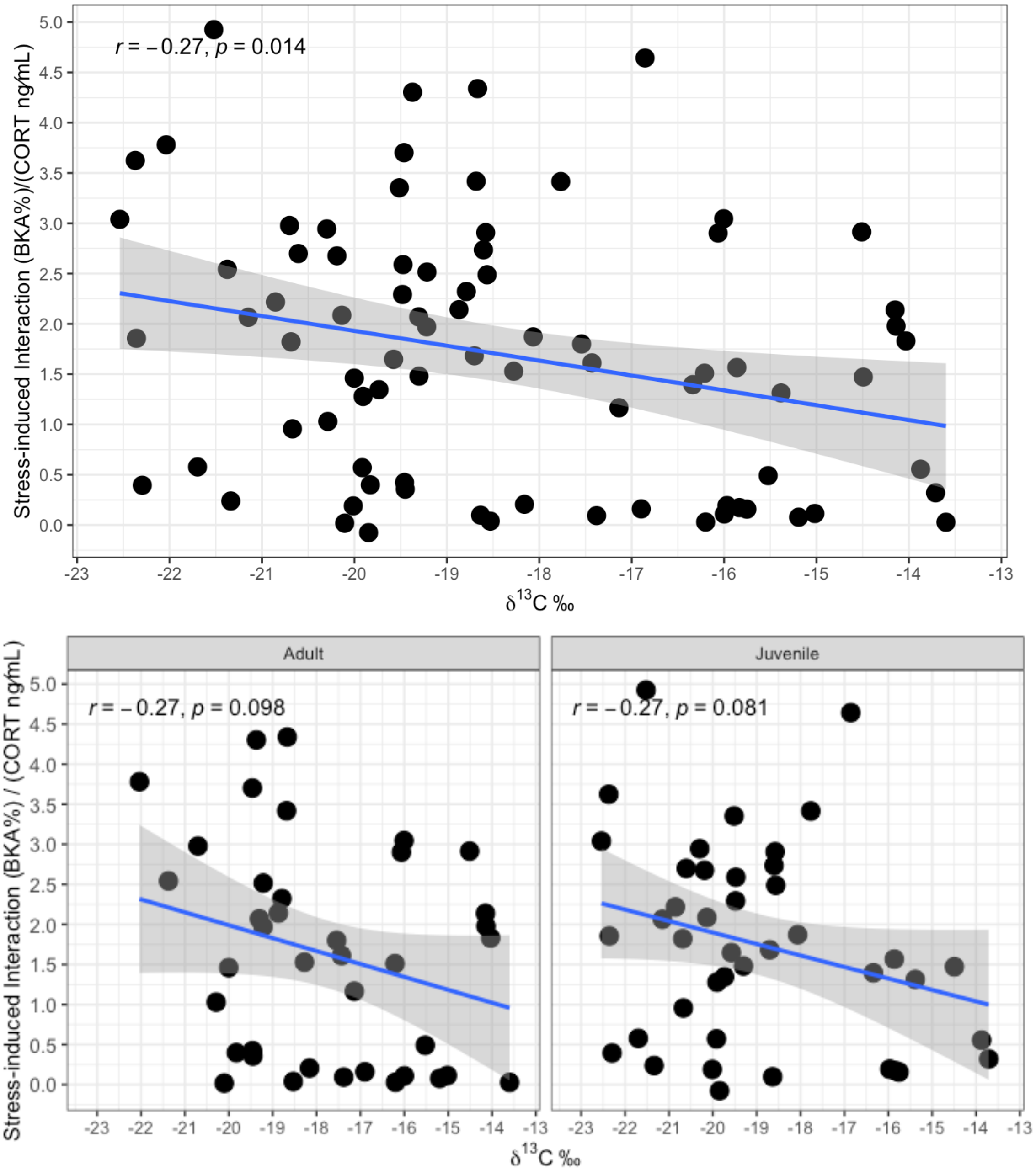


Figure 5.8 (top) Pearson's correlation (r = - 0.27, p = 0.014, n = 82) for the relationship between δ13C ‰ and stress-induced $\frac{BKA\%}{CORT^{ng}/_{mL}}$. (Bottom left) adults (r = -0.27, p = 0.098, n = 40) and (bottom right) juveniles (r = - 0.27, p = 0.081, n = 42). Standard error with 95% confidence interval shaded in gray.

5.4.4 Immunocompetence, diet, and body condition

Birds (n = 93) exhibited varied directions of the net immune response after 30-minutes of constraint (Fig. 5.9a). A plurality (42%) was negative, i.e., a reduced immune response; 28% exhibited no change (immune response remained constant between baseline and stress induced BKA %); and 30% were positive change (enhanced immune response; Fig. 5.9b). After removing 31 birds lacking data for at least one of the four factors in the PCA, the analysis had a sample size of 62 total birds. The first two principal component axes were unable to distinguish among the three directions of immune response, although they explained 74.4% of the variation (Fig 5.10). A simple biplot revealed overwhelming overlap among the three immunocompetence groups (Fig. 5.10), indicating no significant differences among the groups driven by diet and/or body condition.

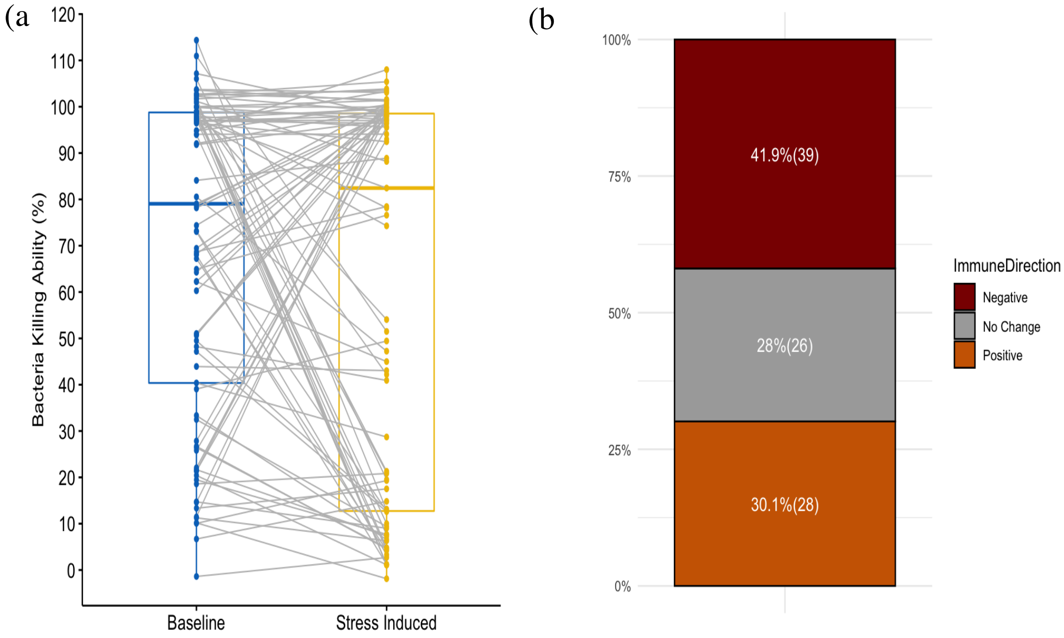


Figure 5.9 (a) Boxplot showing the change in bacteria killing ability % for each individual bird (n = 93) by baseline to stress-induced plasma. (b) Breakdown by percentage and count for the direction of the net immune response (stress-induced BKA % - baseline BKA %).

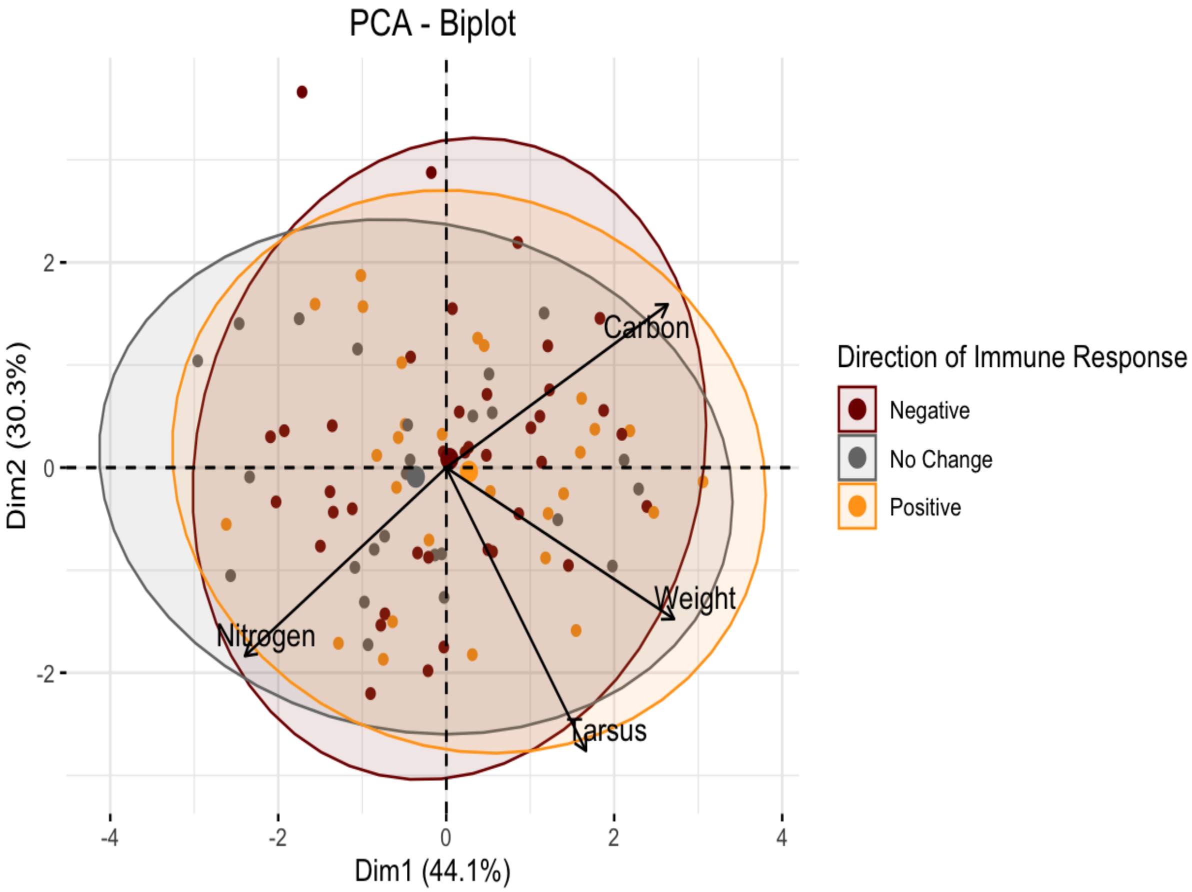


Figure 5.10 Biplot for the direction of immune response. Four features ($\delta^{15}N$ ‰, $\delta^{13}C$ ‰, weight (g) and tarsus length (mm)) used to capture 95% of variability of n = 93 birds. PC1 and PC2 explains 74.4% of variability.

These three immune directions – positive, no change, and negative – were similar for adults and juveniles analyzed separately. For adults, they were 49%, 22%, and 29% respectively (n = 45) and for juveniles they were 35.4%, 33.3%, and 31.2% (n = 48) (Fig. 5.11a). PCAs were repeated to test for effects within adults (n = 27) and juveniles (n= 35) separately, given their different dietary signatures for $\delta^{15}N$ and $\delta^{13}C$ (Chapter 3) and given the tendency for adults to be larger bodied. For both adults and juveniles, principal components 1 and 2 explained 72.49% and

77.12% of the variation, respectively (Fig. 5.11b, c). As before, there were no clear distinctions among the immunocompetence groups for either the adults or juveniles.

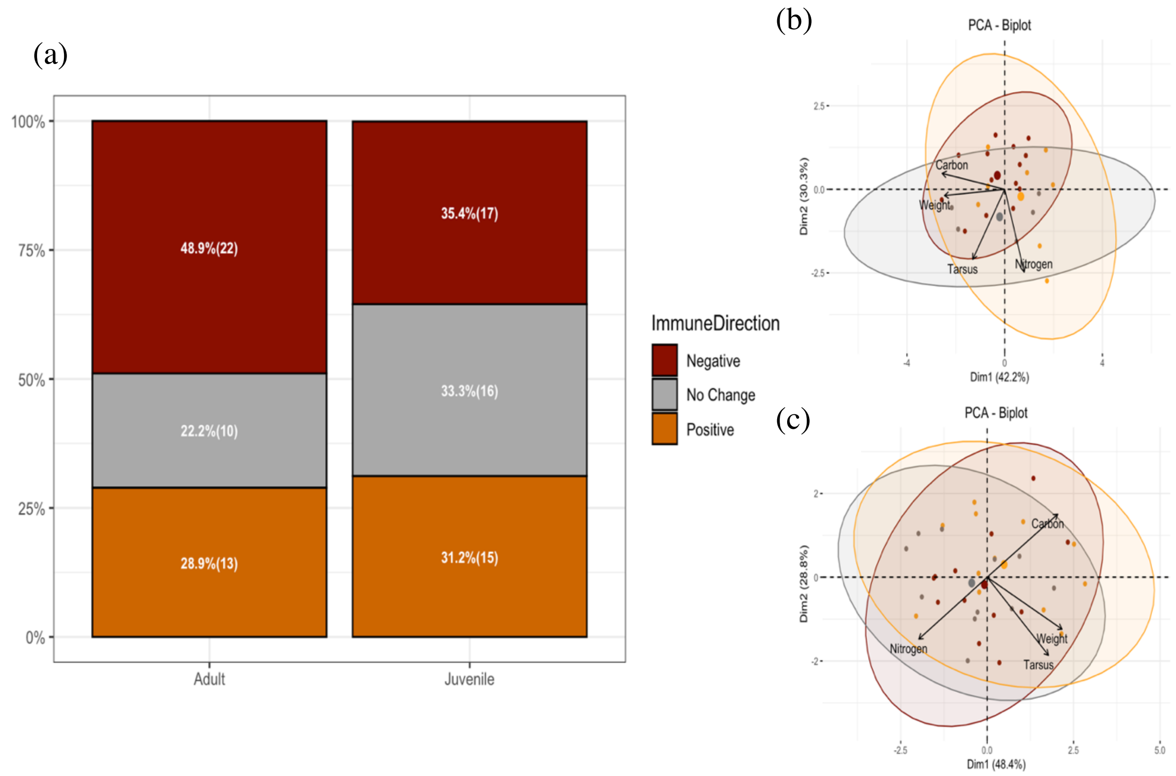


Figure 5.11 (a) Breakdown by percentage and count for the direction of the net immune response (stress-induced BKA % - baseline BKA %) for adults (n = 45) and juveniles (n = 48). (b) biplot for the direction of immune response for juveniles (n = 35). PC1 and PC2 explains 77.12 % of variability and (c) adults (n = 27). PC1 and PC2 explains 72.49% of variability. Four features (δ^{15}N ‰, δ^{13}C ‰, weight (g) and tarsus length (mm)) used to capture 95% of variability of birds.

5.5 Discussion

5.5.1 Corticosterone

In this study, we investigated the stress HPA axis (corticosterone; herein referred to as CORT), innate immune response (bacteria killing ability; herein referred to as BKA), and the interaction between the two systems of house sparrows along an urbanized gradient. We found that CORT levels at baseline, stress-induced or net-CORT did not differ along an urbanized gradient. These results are in align with those found by Chávez-Zichinelli et al. (2010), Bókony et al. (2012), Meillère et al. (2015), and Salleh Hudin et al. (2018). For instance, in Bókony et al. (2012), there was no difference found in baseline nor stress-induced CORT for house sparrows along an urbanized gradient. Additionally, these results were consistent with ours for both juveniles and adults during the non-breeding season although our non-breeding season was defined as September to December while theirs was defined as January to March. Taken together, these results suggest that, at least within the non-breeding season, house sparrows along an urbanized gradient are experiencing their environments similarly. In other words, these results suggest that urban sparrows are not under any more or less stressful conditions than their rural counterparts. However, unlike Chávez-Zichinelli et al. (2010), Bókony et al. (2012), and Meillère et al. (2015), we did quantify dietary signatures and found that more urban birds had significantly lower $\delta^{13}C$ (adults: $r = -0.52$, $p < 0.0001$, $n = 70$; juveniles: $r = - 0.27$, $p = 0.018$, $n = 76$) and higher $\delta^{15}N$ (adults: $r = 0.28$, $p = 0.02$, $n = 70$; juveniles: $r = 0.52$, $p < 0.0001$, $n = 76$) in their diets than less urban birds (Fig. 5.7.1). The results for $\delta^{13}C$ and $\delta^{15}N$, led us to conclude that although CORT responses suggest that house sparrows are under comparable environmental conditions it is unlikely that comparable environmental conditions include access to similar foods. Here we see differences in food exploitation, where less urban birds have higher levels of C4 plants in their diets and are feeding at a lower trophic level than more urban birds, which could be attributed to the increased amount of corn fields found in those areas and reduced insect-rich diet, respectively (however, see discussion section from Chapter 3). On the other hand, similarities in CORT responses could be due to all populations experiencing molt during our sampling time point. Molting was shown to be correlated with reduced CORT responses in house sparrows (L.M. Romero 2002) and therefore it is not unlikely that house sparrows sampled along an urbanized gradient during molt in this study represent the overall effect of molt on CORT responses.

We found a significant positive relationship between stress-induced CORT and $\delta^{13}C$ and net-CORT and $\delta^{13}C$, demonstrating that birds with an enriched C4-plant based diet had a more sensitive stress HPA response to being held in cloth bags. Researchers sometimes consider low CORT levels to be related to high food availability and by extension high-quality foods (Kitaysky, Piatt, and Wingfield 2007; Kitaysky et al. 2010; Barger and Kitaysky 2012), therefore under that interpretation, our results may be capturing that enriched $\delta^{13}C$ diet corresponds to a lower quality diet due to its positive relationship with stress-induced and net CORT. However, we are not confident in that conclusion as Kouwenberg et al. (2016) found that auklets *Cerorhinca monocerata* at a site with poor food abundance had lower CORT than those at the high food abundance site. Contrary to the positive relationship between $\delta^{13}C$ and CORT seen in our current study, Fairhurst et al. (2013) found that CORT (deposited into feathers) was negatively correlated with $\delta^{13}C$ values for Dupont's larks *Chersophilus duponti*. Fairhurst et al. (2013) explained that the negative relationship between CORT and $\delta^{13}C$ was possibly a result of the interaction between increased arthropod abundance and variation in water use efficiency of plants, however this reasoning is not particularly clear to us. However, it was noted that the $\delta^{13}C$

values of arthropods increase with the percentage of C4 crops (Briones et al. 2001). Note: the stomach contents of a few larks suggested that their diet is composed of insects and seeds (de Juana and Suárez 2020).

To further explore the CORT- δ^{13}C relationship further, we decided to analyze the relationship of CORT (both stress-induced and net-CORT) and δ^{13}C by age. Our rationale was that if a positive relationship between CORT and δ^{13}C truly existed then it may be driven by adults or at least be most apparent in adults since their primarily plant-based diet is reflected by δ^{13}C ‰. The relationship between stressed-induced CORT and δ^{13}C was no longer significant, which calls into question the meaningfulness of observing a positive relationship prior, however, to our surprise, juvenile net CORT was significantly correlated with δ^{13}C values. The reason for why net CORT and δ^{13}C were positively correlated for juveniles may be due to the physiological interaction mentioned above: poor quality foods may induce the production of CORT. It is not entirely clear why this effect was not observed in adults.

Lastly, in line with results from Meillère et al. (2015), we also found that baseline CORT did differ by age, where adults had significantly higher baseline CORT than juveniles. The difference in baseline CORT could reflect the allostatic load associated with the different life history stage in house sparrows. Allostatic load represents the cumulative “wear and tear” an organism experiences throughout their lifetime as they adjust their physiology to regain homeostasis through the process of allostasis (reviewed in Ramsay and Woods (2014)). In other words, according to Ramsay and Woods, organisms must regulate their physiologic parameters (here, homeostasis) via adjustments (here allostasis) to cope with changes in their environments. Therefore, the baseline CORT levels observed here may represent the shift from low to high levels as individuals continue to experience more repeated environmental stressors with age.

However, in contrast to our results, Wilcoxen et al. (2011) did find a general negative trend for baseline CORT as male Florida scrub-jays *Aphelocoma coerulescens* aged, however that result was not significant and was only apparent during the breeding season as opposed to the pre-breeding season.

A consistent relationship between CORT and age has been demonstrated for circulating stress-induced CORT response and average CORT deposited into feathers (Lendvai et al. 2015; López-Jiménez et al. 2017) where younger individuals tend to have higher CORT levels than older ones (however see Meillère et al. (2015)). One possible explanation for the age-related difference in stress-induced and net CORT is that juveniles exhibit an “in training” status for their stress HPA axis as they learn to respond appropriately to stressors, while the stress HPA axis of their adult conspecifics have presumably already learned appropriate responses to comparable stressors. Like the above studies, we did observe that juveniles had higher circulating net-CORT than adults (although not significant in our current study) and higher CORT in their feathers (seen in Chapter 4). The ultimate effect of age on baseline, stress-induced, and net- CORT response must be formally tested via experimentation before being conclusive.

5.5.2 Innate immune response

We were able to show that baseline BKA (and not stress-induced or net BKA) was positively correlated with urban score. The positive correlation between baseline BKA and urban score could suggest that urban house sparrows are under selection pressures to mount a more sensitive immune response than rural birds (Moller (2009)). Multiple studies have shown similar outcomes in support of an enhancement of immune activity in urban birds (Audet et al. (2016), and Cummings et al. (2020)). Conversely, as suggested by Peneaux et al. (2021), it is also possible that we have detected a "pre-activated" immune response associated with urban sparrows already combating infections. Urban bullfinches *Loxigilla barbadensis* were reported to have a 2.6-fold stronger T-cell mediated immune response to phytohemagglutinin than rural birds (Audet, Ducatez, and Lefebvre 2016). Similarly, Cummings and colleagues (2020) found that plasma from urban white ibis *Eudocimus albus* had higher *E. coli* killing ability (the same measure of immunity used in our study) than those from rural habitats. A possible reason that urban birds have a more sensitive immune response than rural individuals could be related to their increased exposure to infectious diseases (e.g., bacteria enteropathogens, viruses). In urban areas, high population density and aggregation of wild birds at supplemental food sites (e.g., bird feeders) may contribute to increased transmission of pathogenic microbes (Dhondt, Tessaglia, and Slothower 1998; Bradley and Altizer 2007; Becker, Streicker, and Altizer 2015). For example, because of aggregation at bird feeders, *Trichomonas gallinae* (a protozoal parasite) may have been transmitted from Columbiformes to songbirds, which led to the death of ~1.5 million greenfinches (*Carduelis chloris* and *chaffinch Fringilla coelebs*) between 2007 to 2009 (reviewed in (Lawson et al. 2012)). Empirical evidence from Hamer et al. (2012) showed that wild birds in the Chicagoland area had greater exposure to pathogens as urbanization increased. Therefore, it is quite possible that a positive correlation between baseline BKA and urban score highlights a selection pressure for urban house sparrows to rapidly clear pathogens. However, we do not neglect Peneaux et al. (2021) point that our result may indicate that urban birds have a hyperactive immune response because they were concurrently fighting other infections. To our surprise, the direction of the net innate immunity was heterogenous and presents evidence that there may be population variation that the field has not accounted for. Critically, our results show that immune response is not fixed and may be highly dependent on context. Upon further analysis, we found that variation in net innate immunity was not related to diet nor weight, two indicators of body condition. Although the cause for variation in net innate immunity remains undetermined in this study, future studies should consider identifying whether variation exists within a single sample population. Unfortunately, we were unable to do so in this study due to limited sample size.

We found that CORT was associated with heightened BKA in juvenile birds during the stress-induced timepoint, and not in adults. Specifically, an increase in stress-induced CORT was positively correlated with an increase in *E. coli* killing ability. Similar results were observed in house sparrows studied by Vagasi et al. (2018). These researchers found that the activity of the innate complementary system (measured as hemolysis scores) increased in birds with CORT implants. When baseline CORT and BKA (*E. coli*) were studied in red-winged black birds *Agelaius phoeniceus*, Merrill et al. (2014) also found a positive correlation between them. However, unlike these researchers who identified a positive relationship between CORT and BKA at baseline (blood collected within 3 minutes), we observed a positive CORT-BKA relationship at the stress-induced time point. The difference between our study and (Merrill et al. 2014) may, in part, be a result of the different species and life history stage used in each study.

In opposition to our results, two recent studies in house sparrows (Gao, Sanchez, and Deviche 2017; Gao and Deviche 2019) showed empirical evidence that CORT has an immunosuppressant effect. The results from the two complementary studies showed that when a CORT synthesis blocker, mitotane, was administered, an immunosuppressant effect (here BKA with *E. coli)* was not observed. However, the control birds (individuals who did not receive mitotane) showed a significant decrease in bactericidal ability. An important distinction between our study and that of (Gao, Sanchez, and Deviche 2017; Gao and Deviche 2019) is that our samples were collected in the field while their samples were collected after sparrows acclimated to a lab environment. Researchers have shown that both immune and stress response in house sparrows has the capacity to change with captivity (Martin et al. 2012; Love, Lovern, and DuRant 2017). Therefore, variation in the effect of CORT on innate immunity among studies may be somewhat related to studying animals in the wild versus in the lab.

5.5.3 Interaction between innate immunity and corticosterone

We aimed to get a single value to represent the interaction between CORT and BKA. To our knowledge, this is the first study to account for BKA % and CORT with a single normalized value. We initially hypothesized that values of the interaction term, $\frac{BKA\%}{CORT^{ng}/_{mL}}$, would show a significant relationship with urbanization. We speculated that the heterogeneous results in the literature for CORT and BKA % may be explained by identifying that the two systems interactive differently along an urbanized gradient. In other words, BKA % may be negatively correlated with CORT in rural areas, and positively correlated in urban areas. However, our mixed models suggested that only the stress-induced interaction $\frac{BKA\%}{CORT^{ng}/_{mL}}$ was negatively correlated with $\delta^{13}C$, but the meaningfulness of that result is in question (relationship changed with age) and therefore this section of our study is inconclusive.

5.6 Conclusions and future studies

The primary goal of this study was to determine whether heterogeneity in physiological measurements along an urbanized gradient could be better understood once viewed as subsets of a whole. We hypothesized that the inconsistent results demonstrating the effect urbanization has on corticosterone levels may be related to variations in other interconnected systems such as the immune response. We also assessed corticosterone level and innate immune response by determining the dietary signatures of individuals comprising a population. To some degree, our study does suggest that corticosterone and immunity are interacting in birds along an urbanized gradient, however, substantial evidence must be gathered to properly test this hypothesis.

The most noteworthy result in this study demonstrated that urban habitats could impose selective pressure on house sparrow immune response. We identified that for both adults and juveniles, baseline innate immunity increased with urbanization, which could be related to the increased exposure to infectious diseases often described in urban environments. Additionally, we found that corticosterone levels did not differ along an urbanized gradient, and *that* lack of difference was not reflected in dietary signatures. In other words, although we were able to identify that both $\delta^{13}C$ ‰ and $\delta^{15}N$ ‰ changed with urbanization, we found that baseline-, stress-induced-, and net- corticosterone levels remained similar. One key physiology that could account for our corticosterone result is the gut microbiome. The gut microbiome has the potential to (directly or indirectly) mediate the stress HPA axis and immune system (see Chapter 2 for review). Furthermore, there is evidence showing that house sparrow microbiome can differ along an urbanized gradient (Teyssier et al. 2018; Teyssier 2020).

We acknowledge that our results are limited to a single timepoint and would benefit from longitudinal studies that capture different life history stages over multiple years. An additional caveat is that we did not include multiple measurements of stress HPA activity (e.g., heterophil to lymphocyte ratio) or immune response (e.g., leucocyte counts), which together could have highlighted the trade-offs individuals are making to cope within their habitats. Future studies would benefit from a multifaceted case-control lab study design that is then built upon with field experiments to determine the interaction between corticosterone, innate immune, and gut microbiome along an urbanized gradient.

5.7 Supplementary

Table 5.7.1 Linear mixed model by maximum likelihood explaining variation in baseline-, stress-induced-, and net- bacteria killing ability (BKA %) and corticosterone (CORT ng/mL), and the interaction BKA / CORT $\frac{BKA\%}{CORT^{ng}/_{mL}}$. Models selected by using a stepwise approach starting from full models and removing predictor variables with $P > 0.05$. Bolded values represent $p < 0.05$.

Dependent Variable	Predictor Variable	Estimate	Std. Error	df	t-value	p-value
baseline CORT	age	-3.37	1.49	116.91	-2.26	**0.025**
	urban score	0.63	0.58	12.82	1.087	0.29
	δ^{15}N ‰	0.22	1.19	63.91	0.19	0.84
	δ^{13}C ‰	-0.047	0.32	79.48	-0.14	0.88
stress-induced CORT	age	3.31	2.68	92.82	1.23	0.22
	urban score	1.13	1.42	7.57	0.79	0.44
	δ^{15}N ‰	-2.20	2.30	104.019	-0.95	0.34
	δ^{13}C ‰	1.88	0.57	99.32	3.28	**0.0014**
net CORT	age	7.58	2.50	108.10	3.025	**0.0031**
	urban score	1.14	1.33	7.069	0.85	0.41
	δ^{15}N ‰	-1.93	2.21	99.28	-0.87	0.384
	δ^{13}C ‰	1.92	0.52	93.11	3.65	**0.00042**
baseline BKA	age	-6.89	6.23	103.98	-1.10	0.27
	urban score	6.38	2.22	31.44	2.87	**0.0072**
	δ^{15}N ‰	5.64	5.02	85.71	1.12	0.26
	δ^{13}C ‰	0.20	1.42	101.53	0.14	0.88
	baseline CORT	0.38	0.39	84.34	0.95	0.34
stress-induced BKA	age	1.017	9.08	81	0.11	0.91
	urban score	2.10	3.22	81	0.65	0.51
	δ^{15}N ‰	-4.18	7.43	81	-0.56	0.57
	δ^{13}C ‰	-4.39	1.91	81	-2.29	**0.024**
	stress-induced CORT	0.60	0.30	81	1.97	**0.051**

Table 5.7.1 (Continued) Linear mixed model by maximum likelihood explaining variation in baseline-, stress-induced-, and net- bacteria killing ability (BKA %) and corticosterone (CORT ng/mL), and the interaction BKA / CORT $\frac{BKA\%}{CORT^{ng}/_{mL}}$. Models selected by using a stepwise approach starting from full models and removing predictor variables with $P > 0.05$. Bolded values represent $p < 0.05$.

Dependent Variable	**Predictor Variable**	**Estimate**	**Std. Error**	**df**	**t-value**	**p-value**
net BKA	age	10.30	10.38	73.79	0.99	0.32
	urban score	1.62	3.63	71.26	0.44	0.65
	δ^{15}N ‰	-9.70	9.43	72.00	-1.028	0.30
	δ^{13}C ‰	-0.22	2.90	72.07	-0.077	0.93
	net CORT	0.11	0.36	73.54	0.31	0.75
baseline $\frac{BKA\%}{CORT^{ng}/_{mL}}$	age	-0.86	0.75	82.24	-1.15	0.25
	urban score	0.20	0.26	49.35	0.78	0.43
	δ^{15}N ‰	0.40	0.64	77.27	0.62	0.53
	δ^{13}C ‰	-0.027	0.18	82.18	-0.14	0.88
stress-induced $\frac{BKA\%}{CORT^{ng}/_{mL}}$	age	-0.061	0.28	81	-0.21	0.82
	urban score	0.0058	0.099	72.16	0.059	0.95
	δ^{15}N ‰	0.087	0.22	81	0.38	0.70
	δ^{13}C ‰	-0.13	0.058	77.60	-2.22	**0.02**
net $\frac{BKA\%}{CORT^{ng}/_{mL}}$	age	0.31	0.75	68.16	0.41	0.68
	urban score	-0.032	0.27	37.15	-0.11	0.90
	δ^{15}N ‰	-0.13	0.70	70.67	-0.19	0.84
	δ^{13}C ‰	-0.13	0.21	72.37	-0.63	0.52

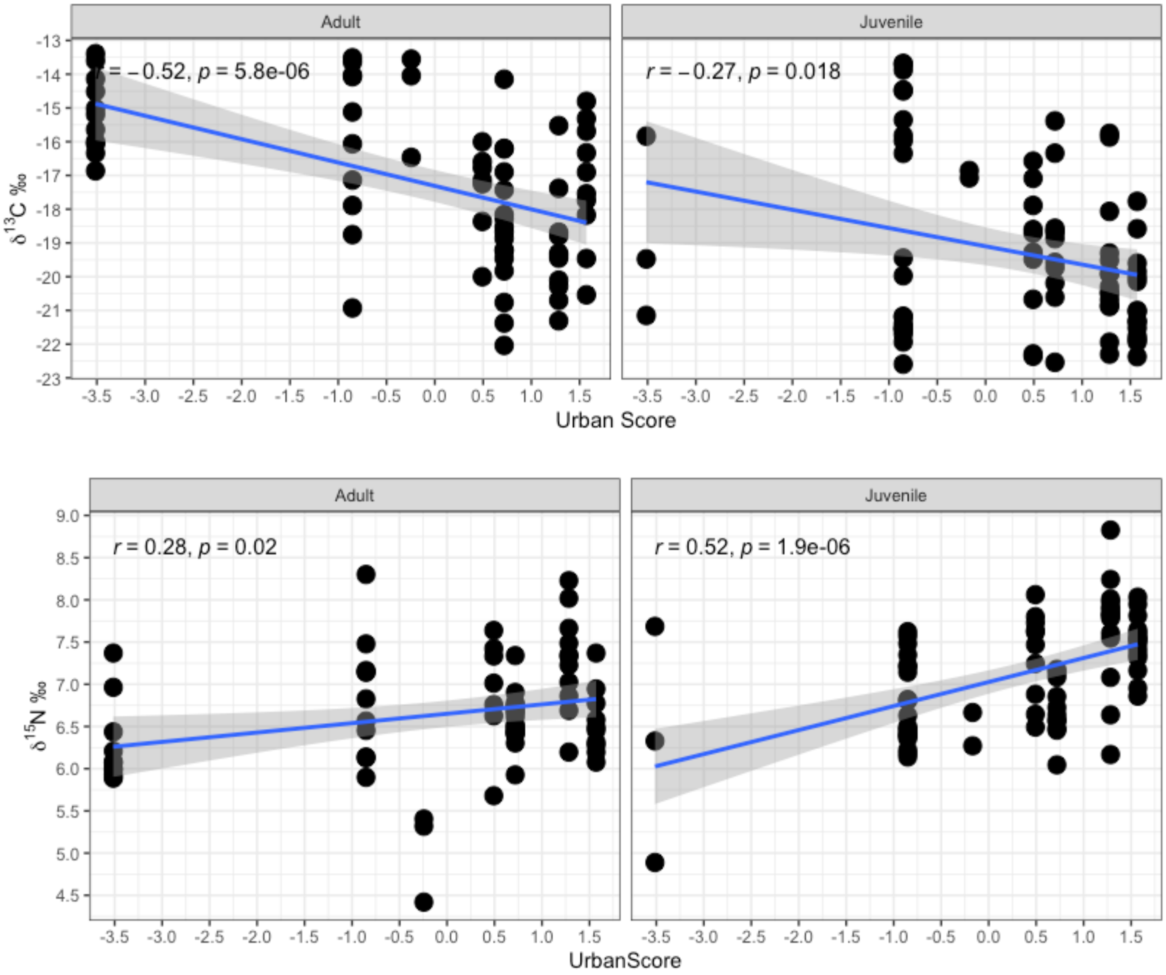


Figure 5.7.1 (top) Pearson correlations between δ^{13}C ‰ and urban score (PC1; logged) for adults (left) and juveniles (right). (bottom) Pearson correlations between δ^{15}N ‰ and urban score for adults (left) and juveniles (right).

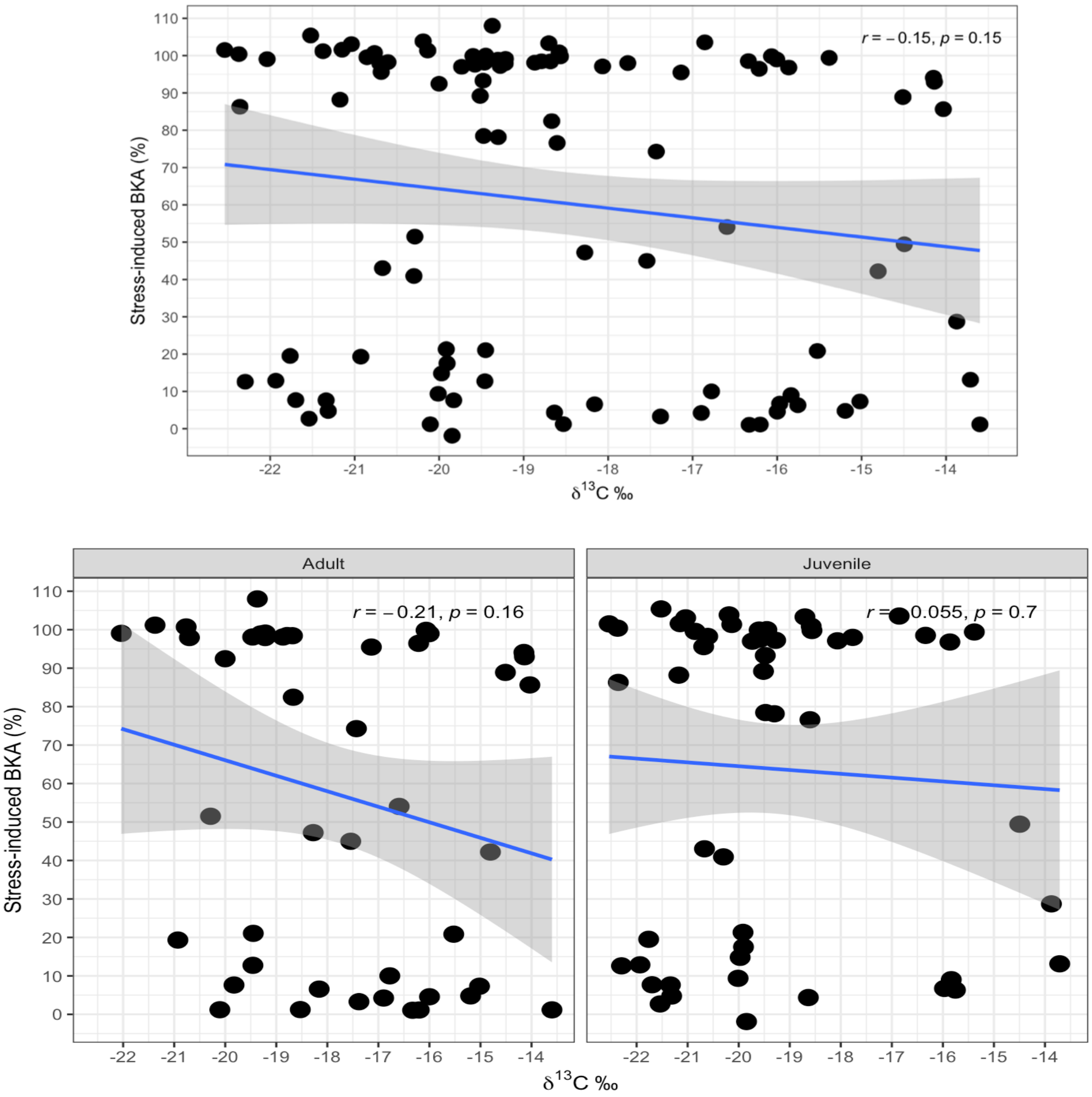


Figure 5.7.2 (top) Pearson's correlation between $\delta^{13}C$ ‰ and stress-induced BKA % for adults and juveniles combined. (bottom) Pearson's correlation between $\delta^{13}C$ ‰ and stress-induced BKA % for adults (right) and juveniles (left).

Chapter 6

Technical pitfalls and potential solutions: a reflection on studying the microbiome of small free-living passerines

6.1 Abstract

This chapter discusses the challenges and pitfalls, encountered during the collection, extraction, and sequencing of microbial DNA (16S rRNA amplicons) from free-living birds. Samples collected included cloacal, eye, mouth, uropygial gland, and feces. I highlight my experiences and concerns for the reproducibility and reliability of avian microbiome studies and calls for the standardization of lab protocols. The goal of this essay is to equip future researchers with knowledge of the limitations of studying the microbiome of wild animals that can yield low-biomass samples, and to suggest solutions that may help to develop this growing field.

6.2 Introduction

The microbiome is an ecological community of microorganisms (bacteria, archaea, fungi, viruses) that play an important role in the health and adaption (Chow et al. 2010; Sharpton 2018; Ortega et al. 2021). Once viewed exclusively as disease-causing agents, microbes can have both symbiotic and pathogenic relationships with their hosts and can affect fitness by modulating nutrient uptake, immunity against diseases, and other physiological systems (Chow et al. 2010; Lee and Mazmanian 2010; Lyte 2010). Diverse communities of microbes have built a relationship with animals – living on their skin, scales, and feathers, as well as in their respiratory, reproductive, and digestive tracts (Chow et al. 2010; Kohl 2012) -- and their role of impacting the evolution of animal-hosts is becoming increasingly clear as more research is conducted in this area. However, the standard practice for collecting, storing, extracting, and computationally examining the microbiome is constantly being revised and updated.

Here I will outline the most recent changes that have occurred in respect to each stage (if any exists) while reflecting on practices that I made during my study on the avian microbiome using free-living house sparrows (*Passer domesticus*). Despite considerable effort, both in the field (e.g., using dry ice to store samples instead of wet ice) and in the laboratory (e.g., multiple independent runs at two different facilities), bacterial biomass yield remained consistently low and noisy due to well-to-well contamination. As I will describe further, low microbial biomass and well-to-well contamination are two plights limiting microbiome studies (Song et al. 2016; Minich et al. 2018; Selway, Eisenhofer, and Weyrich 2020).

6.3 Collection and Storage

We suspect that the collection technique, storage method, and length of storage utilized played a role in the low bacterial biomass that samples returned. Samples were collected using sterile swabs that were then cut and stored in sterile polypropylene cryogenic vials on wet ice in the field. For example, eye and mouth samples were collected using a single dry swab, rotated gently for ~10 seconds. It is possible that this method of collection was ineffective given the particularly low read counts after denoising using DADA2 (e.g., 0 to 303 for eyes, and 0 to 70 for mouth; Fig. 6.1) and Deblur (0 to 188 for eyes, and 0 to 47 for mouth) (See supplementary Dada2-table.qzv and Deblur-table.qzv). One possible solution for increasing bacterial biomass from the eye might be to first dip the swab into sterile tryptase phosphate broth (TPB), as used by Thomason et al (2017). Those researchers followed up with two additional steps: swirling swabs in 300 μL of sterile TPB after swiping the eye and then wringing out the swab to collect the remaining fluid. A similar procedure may prove effective at increasing biomass for the mouth and cloaca (both sample types yield low read counts like those of eyes).

The collection process for feces was different. Like Knutie and Gotanda (2018), an individual bird was secured to prevent flight and then placed into a clean brown-paper bag, which usually resulted in defecation. A subset of any feces was collected into sterile polypropylene cryogenic vials (no swabbing) and then stored on ice. The read count for feces samples showed high variation, from 0 to 107, 483 (Fig. 6.1). However, we suspect contamination given that most blank samples (i.e., sterile DNA-free water) did not have low reads but instead ranged from 26 to 30,026 (See next section: microbial DNA extraction).

All samples were placed into a cooler with ice packs (frozen at a -80° C) and stayed in the cooler for up to 5 hours until they were transported to a -20° C freezer. These samples stayed in the -20° C freezer for up to 7 – 9 months until DNA extraction. We have identified two key pitfalls associated with storing these samples that may have contributed to the unreliable 16s rRNA amplicon sequences in our study. Firstly, due to the logistics of field research, we were not able to take samples to the -20° C freezer within a more reasonable time. Future researchers who insist on storing samples with only freezing as a means of preservation should consider limiting the time of field collection (to quickly return to the laboratory) and bringing dry ice. Secondly, many samples were stored in freezers at a steady low temperature for several months (e.g., 7 to 9 months). Long-term storage likely contributed to the degradation of samples. Song et al. (2016) presented two possible solutions to overcome these limitations: (1) using a stable preservation method such as 95% ethanol, FTA cards, or a OMNIgene gut kit, and (2) analyzing samples within eight weeks of the date of collection. We highly recommend the use of Whatman FTA cards for field work. FTA cards are convenient for field studies because they allow samples to be stored at room temperature with some confidence that sample DNA will be trapped and preserved in the FTA card matrix. Furthermore, Song et al. (2016) found that FTA cards tend to recover a greater diversity of bacterial taxa than other preservation methods, which may be due to the lysing chemistry on the card.

Figure 6.1 Boxplot of DADA 2 read features for processed samples. Blank (n =39) refers to negative controls, i.e., PBS; Zymo mock (n = 32) community refers to the positive control samples. For the dissertation, samples were collected from eyes (n = 20), mouth (n = 20), cloaca (n = 73), uropygial gland (n = 63), feces (n = 142), gizzard (n = 19), lower intestine (n = 22), middle intestine (n = 22), and upper intestine (n = 23).

6.4 Microbial DNA extraction

We extracted microbial DNA using 16S Illumina Amplicon Protocol used by the Earth Microbiome Project (https://earthmicrobiome.org) at the University of Chicago and at the University of California San Diego. The Qiagen MagAttract Kit was used with a few modifications to improve extraction yield (Trevelline et al. 2018). For instance, we increased the length of the incubation period to overnight for step 5 as recommended by Trevelline et al. (2018). We also added positive and negative controls to our 96-well plates to test for possible well-to-well contamination. Negative controls were sterile DNA-free water, which should have little to no DNA, while the positive controls were serially diluted Zymo Mock communities and should show a sequential change in DNA concentration.

We highly recommend using positive and negative controls on each plate as a part of the standard lab protocol to determine extraction efficiency, i.e., a clear baseline. The first two iterations of DNA extraction in our research did not include sufficient negative controls, and we did not have any positive controls. These controls would have likely saved a lot of time. Namely, there are baseline results expected for negative controls and serially diluted positive controls, where blank samples should have little to no DNA, and positive samples should show a sequential change in DNA concentration. If the results from a PicoGreen assay, an assay used to quantify DNA, deviates from the baseline expectation, then one has an immediate alert to reexamine the extraction process including checking the expiration dates of plates, kits, and primers. If, on the other hand, PicoGreen results are as expected, they may draw attention to low reads associated with low biomass samples that require intervention.

We also recommend maintaining an “in house” log of the PicoGreen results for the control. For example, there should be a lab-associated range, mean, and standard deviation that is continuously updated after each extraction. Such a log would allow researchers to identify irregularities based on the historic results from the lab. For additional information on the importance of adding controls, please refer to Eisenhofer et al. (2019).

For researchers working with low biomass samples, we also recommend utilizing a bioinformatics technique called KatharoSeq, created by Minich et al. (2018). KatharoSeq is described as a more reliable and time-efficient alternative to single-tube extraction. It utilizes positive (known microbes making up a sample) and negative controls to differentiate a true positive signal in samples with as few as 50 to 500 cells. For example, using the KatharoSeq methodology, we were able to detect and identify contaminants that needed to be removed, and to generate a criterion for sample exclusion. With software assistance from Dr. Minich, we ran our samples using KatharoSeq with useful results (Fig. 6.2). For example, the threshold for sample exclusion was determined to be 22,000 reads: "Typically, the green circles at around 10,000 reads pictured here should titrate back to 500 reads off the 20,000 read mark. Based on this, a sample would need to have ~22,000 reads to pass quality control" (Minich, email communication, March 2021). Our results thus showed a significant amount of noise, i.e., many samples with values between zero and 22,000, and many of our negative controls had uncharacteristically high read counts, well above zero (see Fig. 6.1). Dr. Minich attributed the high read counts seen in the negative controls to well-to-well contamination, where DNA from the positive controls contaminated nearby wells including the negative controls. This contamination likely occurred during the extraction process. Note: with a criterion of 22k reads, only 11 out of the total 308 samples were usable.

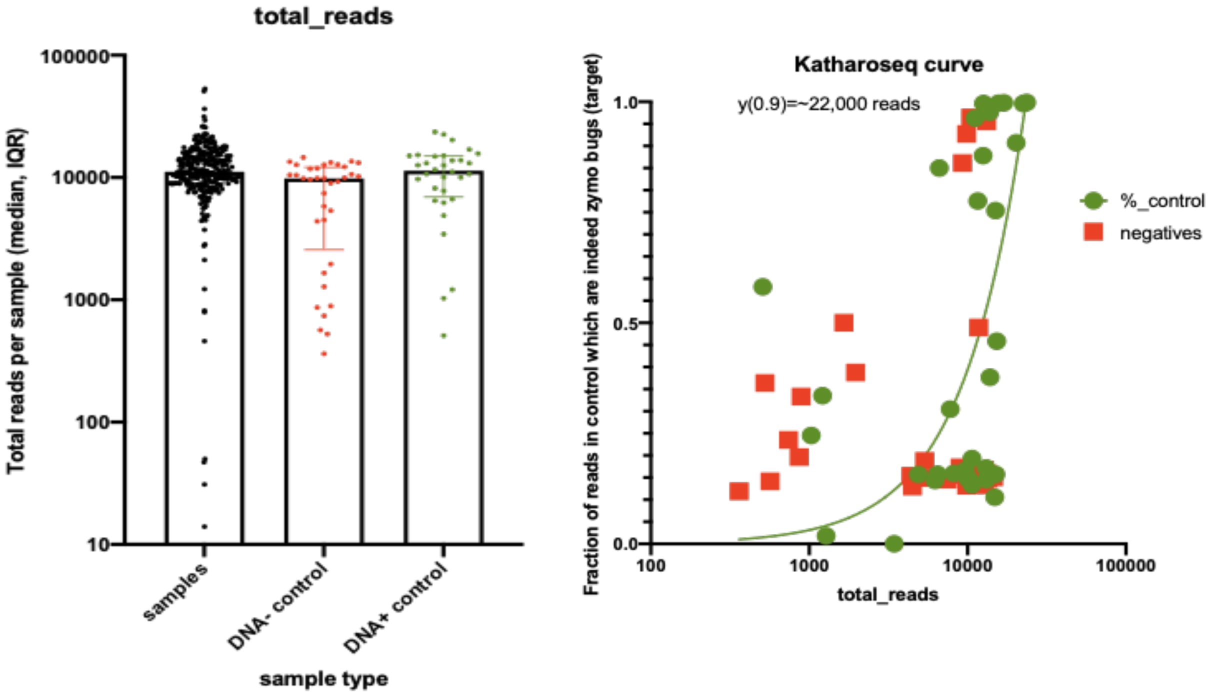


Figure 6.2 (left) Read distribution for avian samples (n = 308), negative controls (n = 36), and positive controls (n = 32). (right) Katharoseq cutoff curve with suggested cutoff of 22k reads. *Credit: Dr. Jeremiah Minich.*

6.5 Conclusion

There is increasing interest in understanding the role that the avian microbiome plays in helping individuals – and populations – cope with the challenges and capitalize on the opportunities that urban habitats present (Phillips, Berlow, and Derryberry 2018; Teyssier et al. 2018; Teyssier et al. 2020). Researchers are actively trying to investigate the effects of the avian microbiota (particularly gut microbiota) on immunity, nutrition, and stress regulation. As research in this area continues, the standards must be reevaluated to encourage accurate microbial representation and reproducibility.

I highlighted a few accessible practices that can help standardize sample storage and extraction. First, storing samples on (e.g., FTA cards) or within (e.g., 95% ethanol) media that can proactively preserve DNA, rather than simply stabilize it (e.g., standard freezing) is the first step to reduce the inconsistencies. Second, limiting the amount of time between sample collection and extraction is also important. We did not have a consistent timeframe for extracting and sequencing samples, which I believe also contributed to the inconsistencies we observed. Third, both positive and negative controls should be added to each extraction plate. These controls will help monitor common issues, such as contamination. They also permit challenges of low biomass from poor preservation to be detected, and resolved, more readily. Such improvements would save a lot of time, money, and resources. Most importantly, pushing the field forward will require clear data to establish accuracy and confidence that samples are truly representative of microbes on which conclusions can be drawn.

Chapter 7

Conclusion and future research

As mentioned in the general introduction: one large source of human-induced change is the rise in urbanization, which is defined as the conversion of natural habitats into areas partly covered by buildings, roads, sidewalks, and other impervious cover that makes an area suitable for permanent and high-density human occupation United Nations (2018). The largely irreversible transformation of both habitat structure and ecological processes in urban areas is expected to act as a set of selective pressures, favoring organismal traits that are best suited to persist or even thrive near humans. These pressures mean that, to forestall local and perhaps permanent extinction, wildlife must adapt to these new conditions, such as through phenotypic plasticity or inter-generational microevolution.

Throughout this dissertation, I focused on understanding the impact of urbanization on house sparrow physiology, namely the stress mediated hypothalamic-pituitary-adrenal axis (HPA), the innate immune response, and the gut microbiome. Additionally, I determined the role that diet, an extension of urban-rural environments, has on stress HPA axis and the innate immune response. The first study showed the reliability of measuring stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) from feathers that underwent the methanol-based corticosterone (herein, CORT) extraction process. Usually, feather pellets are discarded after CORT extraction. Throwing feather pellets away results in the loss of pertinent dietary information that can add another layer to understand how organisms are interacting with their environments. We were able to prove that the methanol-based CORT extraction process does not affect stable isotopic values for $\delta^{13}C$ and $\delta^{15}N$. Moreover, we provided evidence that supports the biological relevance for using the post CORT extraction feather pellets to measure $\delta^{13}C$ and $\delta^{15}N$. We were able to discriminate the insect-rich diet in juveniles (significantly higher $\delta^{15}N$) from the plant-rich diet in adults (significantly higher $\delta^{13}C$) (Fig. 3.2). Together, these results highlight the opportunity for urban ecologists to quantify multiple biomarkers without the limitation of not collecting enough sample mass or volume (e.g., having a sufficient volume of blood to run two separate assays needed to quantify CORT and stable isotope ratios). Using the "one sample two biomarkers" technique can also reduce invasive sampling (e.g., blood and gut contents) and/or eliminate the need for quick and special storage (e.g., sub-zero freezers to stabilize blood, feces, gut contents) associated with field ecology. Because it is still unknown how CORT and stable isotopes are deposited and stored in feathers, future studies should conduct controlled laboratory experiments to test the hypotheses that 1. CORT and stable isotopes are in fact concurrently deposited into feathers and therefore represent the same timeframe; 2. CORT and stable isotopes are trapped in the feather matrix and do not move throughout the feather overtime, including after feather growth (we suggest the use of fluorescent tags); 3. Feathers can reflect seasonal changes in CORT and diet because of the replacement of feathers in between molt. Testing these hypotheses can inspire confidence in the biological relevance of feathers on individual health status or highlight a revision of the ecological tools currently in usage to determine how individuals are responding to their environments.

The second study implemented the "one sample two biomarkers" technique to investigate how CORT, diet, and the interaction between the two effect the body condition of house sparrow individuals along an urbanized gradient. We hypothesized that CORT and diet will affect body condition, and that the bidirectional interaction between CORT and diet is relevant for interpreting body condition. Here, we found that CORT did not affect the body condition of sparrows in urban-rural populations and that CORT did not interact with diet to influence body condition. However, we were able to show that diet, particularly the consumption of C4-enriched foods (e.g., corn), was negatively correlated with body condition. In other words, birds with greater $\delta^{13}C$ values had lower body condition and smaller body mass. Even more exciting, we were able to show that sparrows in rural habitats consumed more C4-enriched foods than urban birds, and that rural sparrows had lower body condition and smaller body mass than their urban counterparts. Rural house sparrows were captured from agricultural land spaces with corn fields and domestic livestock, which most likely contributed to their high $\delta^{13}C$ values. Urban sparrows, however, were captured from a unique type of urban habitat, a zoo in the city of Chicago. We speculate that the greater body condition and larger body mass in urban sparrows may be related to their access to a wide range of foods that could meet nutritional needs. House sparrows captured at the Lincoln Park Zoo had access to the feed set out for animals in captivity (e.g., fish, mice, ribs, and suet and grain mixer). It is possible that zoos around the world could act as a buffer against the variables contributing to worldwide house sparrow population decline. Zoos can provide access to ample nesting sites that are coupled with nutritional food resources and protection against predators. Future studies should investigate whether zoos are an actual “safe haven” that can protect wildlife species from the strains of urban challenges. In other words, test whether wildlife individuals with access to zoo dietary resources can counteract the effect of increased exposure to infectious diseases, heat-island effect, and pollution.

The third study employed experimental ecology to test stress reactivity and innate immune response independently and as interacting systems within different urban settings. We found results that suggest CORT can increase the innate immune response of wild sparrows, however supportive evidence was only found for juveniles. Baseline-, stress-induced-, and net-

CORT did not vary with the level of urbanization. One possibility is that urban and rural birds are experiencing their environments similarly. Interestingly, diet did vary with urbanization. We found that urban birds had significantly lower C4-enriched and higher nitrogen enriched foods in their diets than rural birds. The difference in dietary signatures could indicate that perhaps, CORT levels were not affected by access to different diet resources. We found that baseline innate immunity was positively correlated with urbanization. For both juveniles and adults, as urbanization increased so too did their ability to clear the pathogen, *E. coli*. The positive correlation between urbanization and innate immunity may indicate that urban individuals are under a greater selective pressure to combat exposure to infectious diseases than their rural conspecifics. Studies have reported that a potential challenge in urban ecosystems is the probability of being infected with a pathogen. Large population densities in urban areas increase the rate of transmission for infectious diseases. Particularly as individuals aggregate around supplemental food resources like bird baths and feeders. Urban birds having enhanced immune response to rapidly clear pathogens is a good indicator that wildlife species, whether through plasticity or genetic changes, can cope with challenges in urban habitats. Future research should determine whether an increased immune response comes with a cost to other physiological systems for urban birds. Admittedly, parts of our study remain inconclusive. For instance, we were not able to test interaction terms in our linear mixed models, which limited our understanding for how pairwise interactions among age, sex, diet, and weight influenced CORT and innate immunity. We also failed to determine whether the interaction between CORT and innate immunity differed with urbanization. The interaction term we calculated for the two physiologies, bacteria killing ability percentage divided by CORT concentration, may have been too rudimentary in its approach and requires future revisions.

Altogether, this dissertation highlights the complexities associated with studying urbanization in wildlife animals, particularly how urbanization affects physiology. The complexity increases as researchers begin to ask questions about how different physiological systems are interacting with one another given the evolutionary history of shared bio-mediators and receptors. There is growing evidence that physiological systems are constantly interacting to maintain homeostasis and help organisms cope with perturbations. In the introduction of this dissertation, we speculated that the interdependent connection among three systems, stress HPA axis, immune response, and gut microbiome may widen the possible phenotypic responses wild animals can mount to cope with urbanization. We vehemently suggest that urban ecologists collect multiple biomarkers and conduct nuanced experiments to decipher how physiological systems interact with each other under urban pressures and opportunities.

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