Effects of urbanization on the physiology, behavior, and fitness of a wild songbird

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biological Sciences

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08/19/2022
Blacksburg, VA

Keywords: urbanization, aggression, parental care, glucocorticoid receptors, neurophysiology

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ABSTRACT

As urbanization spreads, understanding its impact on wildlife is increasingly urgent. By comparing the traits and fitness of individuals within the same species found in both urban and rural habitats (urban adapters), we can better understand the behavioral and physiological coping mechanisms wild birds employ in the face of rapid environmental change. For my dissertation, I investigated the physiological, behavioral, and fitness differences between urban and rural living song sparrows (*Melospiza melodia*) to explore how song sparrows are adjusting to urban environments. In my first chapter, I investigated urban birds' termination of the glucocorticoid stress response by looking at their ability to reduce circulating levels of glucocorticoid 'stress' hormones and the relative abundance of neural receptors that provide negative feedback. I found that urban males have a lower relative abundance of glucocorticoid receptors and the enzyme 11β-HSD2 in the hippocampus compered too rural. In chapter 2, I asked if increased aggression, which has been rigorously documented in urban males, is also expressed by females. Indeed, female song sparrows expressed increased aggressive signaling compared to rural, suggesting urban habitats may favor a more aggressive phenotype. Finally, in Chapter 3, I investigated the consequences of increased male aggression on their social partners and offspring by measuring parental care and nestling outcomes across urban and rural habits. I was unable to establish a trade-off between parental care and aggression in either sex. In fact, the more aggressive urban males visited the nest significantly more frequently, a trend also seen in urban females during the daylight hours. Urban birds also had significantly higher reproductive metrics compared to rural, though they also had the added cost of increase brood parasitism compared to rural. Overall increased urban aggression was associated with higher reproductive success without any reduction in paternal care. Additionally, we found physiological differences in the glucocorticoid stress response system associated with the differences in habitat but whether these differences represent mechanisms of acclimation or potential costs of living in urban habitats is not yet clear.

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GENERAL AUDIENCE ABSTRACT

As urbanization spreads, understanding its impacts on wild bird conservation is increasingly urgent. By comparing the behaviors and reproductive success of animals living in urban and rural habitats (urban adapters), we can better understand the coping mechanisms wild birds' employ in the face of this form of rapid environmental change. In my dissertation, I compared the physiology, behavior, and reproductive success of urban and rural song sparrows (*Melospiza melodia*) to explore the changes song sparrows make to live in urban environments. In my first chapter, I investigated how urban birds terminate their stress response by looking at their ability to reduce circulating levels of stress hormones and the relative abundance of "shut down" targets in the brain. In chapter 2, I asked if increased aggression, regularly document in urban males, is also expressed by females. Finally, in Chapter 3, I investigated the consequences of increased male aggression on their social partners and offspring by measuring parental care and nestling outcomes across urban and rural habits. I found that urban males have a lower relative abundance of one type of "shut down" target, and a lower abundance of a potentially protective enzyme in the hippocampus, though we found no difference in how quickly urban and rural birds cleared stress hormone from their blood. Female song sparrows expressed increased aggressive signaling compared to rural, suggesting urban habitats may favor a more aggressive pattern of behavior. However, I was unable to establish a trade-off between parental care and aggression in either sex, suggesting increased aggression is not constraining other reproductive behaviors. In fact, the more aggressive urban males visited the nest significantly more often. Additionally, urban birds had significantly higher reproductive metrics compared to rural, though they also had the added energetic cost of increased brood parasitism compared to rural. Collectively, my results suggest that song sparrows may benefit from livening in low intensity urban habitats and that living in such altered environments favors or permits higher aggression.

DEDICATION

To Patti Lane for always cheering me on.

To Doc Lane for teaching me to question everything.

Acknowledgments

I would first like to thank my advisor, Kendra Sewall, for being a truly spectacular mentor and person. Thank you for always pushing me, for giving me the space to flounder and make mistakes, but with the support to never be hindered by them. Thank you for creating a collaborative, creative environment full of wonderful people and endless opportunities to explore science. I could never have imagined all the amazing opportunities, friendships, and experiences that would stem from a single choice, but joining your lab will always be one of the best decisions I have ever made.

I would also like to thank my advisory committee Bill Hopkins, Joel McGlothlin, and Ignacio Moore for all the feedback, advice, and guidance. I was lucky to have such a cohesive, dynamic, and brilliant group of scientists to learn from. I would also like to give special thanks to Ignacio for being my letter writer on many (often last minute) grant and job applications, and for always being someone I could bounce ideas off of or get quick advice while passing in the hallways.

Thank you to all my field crews, and to the many graduate students who helped me across the 6 truly brutal field seasons; especially to Valerie Brewer and Courtney Linkous, for setting the bar so high as research techs and teaching me so much about mentorship. Finally, thank you to Isaac VanDeist for white knuckling the COVID year with me, and for being an amazing friend and lab mate, I could not have done it without you.

I would also like to thank my mentors from my time as an undergraduate in the Deviche lab at ASU. You were my introduction to science and let me find my passion in life. I would never have made it to this stage of my life and career without you taking a chance on me.

To all the friends I have made during my time at Virginia Tech, but especially to my cohort: thank you for all the vent sessions, the lunches, and the adventures, I wouldn’t have made it without you all. Thanks to David Millican for being my friend and roommate for 6 years, you were always there to lean on in the weird, dark times of grad school.

Thank you to Taylor Fossett for finding me, for being the most wonderful and kind human being, and for all the love and support you have given me. <3

Thank you to all my friends from the creek, in the long, long ago, the before times. Especially to Matt and Wes, for putting up with so many phone calls, texts, and memes when I was missing home.

Finally, I would like to thank all my family that have been so wonderful and supportive. Especially to my mom, who has always been my biggest fan, and to my dad, for being a sounding board for strings of nonsense ideas. Thank you both for your endless support and love over the years.

Attribution

Chapter 1 was coauthored by Michael G. Emmerson, Isaac J. VanDiest, Catherine Hucul, Michelle L. Beck, Scott Davies, Elizabeth R. Gilbert, and Kendra B. Sewall. **Michael G. Emmerson:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - review & editing. **Isaac J. VanDiest:** Methodology, Investigation, Writing - review & editing. **Catherine Hucul:** Methodology, Investigation. **Michelle L. Beck:** Investigation, Writing - review & editing. **Scott Davies:** Investigation, Writing - review & editing. **Elizabeth R. Gilbert:** Resources, Supervision, Writing - review & editing. **Kendra B. Sewall:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Project administration, Funding acquisition All coauthors provided comments and approved the manuscript.

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Introduction

Urbanization is changing habitats globally, and this form of human-induced rapid environmental change (HIREC) is likely to continue to increase in years to come (Sih 2013; Sih et al. 2016). Understanding how animals can cope with HIREC is important to maintaining urban biodiversity, will give us insight into future ecosystems, and help protect vulnerable species (Elmqvist et al. 2013). Challenges to wildlife imposed by HIREC include human disturbance, artificial light at night, increased ambient noise, habitat fragmentation and degradation, and altered anthropogenic predator communities (Injaian et al., 2018; Kleist et al., 2018; Loss et al., 2013; McKinney, 2006; Navara and Nelson, 2007; Rosenberg et al., 2019). Species are generally grouped into three categories based on their ability to tolerate HIREC (Sih et al. 2011; Shochat et al. 2006). Urban avoiders are species that tend to avoid urban areas entirely, whereas urban exploiters are species that almost exclusively live in urban habitats in lieu of their native habitats (Sih et al. 2011; Blair 1996; McKinney 2002). Finally, urban adapters vary within species in their response to HIREC, with some individuals from the same population residing in urban habitats and others in native (rural) habitat (Sih et al. 2011; Blair 1996; McKinney 2002). Within species comparisons of individuals breeding in urban and rural habitats can allow us to understand the acclimations they are making to urban environments. Animals in urban habitats exposed to novel stressors can mitigate them through changes in physiological and behavioral mechanisms underpinning acclimations.

One important and commonly studied physiological coping mechanism is the glucocorticoid stress response. Baseline levels of glucocorticoids are maintained throughout the body by reciprocal communications between the hypothalamus and higher affinity mineralocorticoid receptors (MR) and lower affinity glucocorticoid receptors (GR) (Dickens et al., 2009; Kadhim et al., 2019; McEwen, 2001; Jacobson and Sapolsky, 1991; Sapolsky et al., 2000; Vandenborne et al., 2005; Zimmer and Spencer, 2014). When an external or internal stressor is registered by the peripheral sensory system, signals are sent to the hypothalamus then to the anterior pituitary and finally to the adrenal cortex to then upregulate the production of glucocorticoids (Romero et al., 2009; Sapolsky et al., 2000; Siegel, 1980). This metabolic cascade initiates a suite of behavioral and physiological mechanisms to recover homeostasis (Angelier & Wingfield 2013). However, an important and understudied aspect of this response is its termination, as chronic activations has been linked to a host of debilitating health risks (Dickens et al., 2009; Lin et al., 2004; Lynn et al., 2010; McEwen, 2006; Sapolsky et al., 2000). When glucocorticoids begin circulating at elevated levels, binding of the low affinity GR increases significantly (Dickens et al., 2009; Kadhim et al., 2019; McEwen, 2001; Jacobson and Sapolsky, 1991). This increased binding of GRs sends negative feedback signals to the hypothalamus, and indirectly through the hippocampus, to shut down the response (Sapolsky et al., 2000; Vandenborne et al., 2005; Zimmer and Spencer, 2014). Additional supportive enzymes like 11β-hydroxysteroid dehydrogenase Type 2 (11β-HSD2) (McEwen, 2001; Pérez et al., 2020; Rensel et al., 2018; Seckl, 1997) modulate the effectiveness of glucocorticoids by converting them to inactive forms as they enter the cell, before they can bind GRs (Rensel et al., 2018). An efficient negative feedback system may facilitate colonization of habitats with persistent novel stimuli, such as urban habitats. Animals in urban habitats likely have alterations to their ability to regulate the termination of the glucocorticoid stress response that are paired with more rapid acclimations to HIREC, such as changes to behaviors.

Tone of the first responses an individual can make to HIREC are changes in behavior (Sih 2013; Sih et al. 2016). Indeed, a well-documented behavioral shift between urban and rural animals across taxa, but especially in songbirds, is elevated territorial aggression (Fokidis et al. 2011; Hardman and Dalesman 2018; Ripmeester et al. 2010; Scales et al. 2011). Urban male songbirds that defend seasonal breeding territories have been shown to be more aggressive towards sham and live conspecifics compared to their rural counterparts (Evans et al. 2010; Fokidis et al. 2011; Foltz et al. 2015). Though often overlooked, female songbirds in the north temperate zone can also contribute to territorial defense (Cain and Ketterson 2012; Clutton-Brock and Vincent 1991; Elekonich 2000; Griffith et al. 2002; Stutchbury and Morton 2001; Wingfield 1994). These aggressive female behaviors facilitate resource defense, nest and offspring defense, and work to ensure partner fidelity (Cézilly et al. 2000; Cristol and Johnsen 1994; Heinsohn et al. 2005; Marzluff and Balda 1988; Pärn et al. 2007; Prosen et al. 2004; Reichard and Boesch 2003; Sandell and Smith 1997; Wischhoff et al. 2018). Consistent differences in male aggression between urban and rural habitats suggest that some aspect of urban habitats select for a more aggressive phenotype and territorial females in urban habitats may also benefit from higher aggressive signaling. However, though these changes in aggressive behaviors may facilitate acquisitions of urban territories, they may also constrain other costly behaviors, such as parental care.

Behavioral trade-offs in the breeding season often occur between life-history traits that are constrained by the time and energy available to an individual (Reznick et al. 2000; Roff and Fairbairn 2007; Stearns 1992). Habitat type can modulate these behaviors because an animal’s habitat determines the resources and energy available to them (Gunnarsson et al. 2005; Holtmann et al. 2017). In biparental, territorial songbirds, trade-offs often occur between parental care and other breeding behaviors. In species that express social partnership but genetic polygamy, paternal behaviors can be decreased in favor of extra-pair mating’s or territorial defense (Arcese

1989; Griffith et al. 2002; Møller, 2000). The degree to which these opposing behaviors are constrained or facilitated is determined by the habitat in which they reside. In urban habitats, where evidence suggests elevated aggression is required to obtain a territory, decreases in parental behavior may be required to maintain territoriality and may come at a cost to offspring outcomes (Clutton-Brock et al. 1982; Duckworth 2006; Stearns 1992; Stoehr and Hill 2000). However, in social monogamous species, social partners can coordinate behaviors, and further offset trade-offs imposed by resource availability, or the stressors present in habitats (Duckworth 2006; Marzluff 2001; Scolozzi and Geneletti 2012). Previous research has found that rural house wrens coordinated parental care behaviors more than suburban pairs (Baldan and Ouyang 2020) but few other studies have examined this relationship, and including species with differing life history traits can help us understand if this is a wide spread pattern.

Song sparrows (*Melospiza melodia*) are a common North American urban adapter that establish seasonal breeding territories in both urban and rural habitats where they provide biparental care to a clutch of young (Nice 1943a; Nice 1943b). Both males and females provide territorial defense and are socially monogamous but genetically polygamous, with 12-40% of offspring are sired by extra-pair mates (Keller 1998). As with most North Temperate songbirds, song sparrow females provide most of the parental care through nest building, egg laying, incubation, brooding, and nestling provisioning (Smith et al. 1982). Males provision nestlings then take over the parental care duties after the offspring have fledged so females are free to begin another breeding attempt (Smith and Roff 1980; Smith et al. 1982). Since song sparrows are a biparental, territorial urban adapter (Davies et al. 2016; Evans et al. 2010; Foltz et al. 2015), they are an excellent candidate to study the effects of urbanization on behavioral trade-offs during the breeding season and the physiology that underpins their ability to inhabit urban areas.

Chapter objectives

Across the three chapters of my dissertation, I aimed to explore the effects of urbanization on the physiology, behavior, and the consequence of these differences for mates and their offspring in urban and rural song sparrows. In my first chapter, I investigated the regulation of glucocorticoid negative feedback across urban and rural male song sparrows. I wanted to see how quickly urban and rural birds could reduce circulating glucocorticoids, and the differences in neural mechanisms of the hippocampus and hypothalamus that regulated negative feedback. In my second chapter, I asked how urban female song sparrows alter their levels of aggressive signaling compared to rural. It is commonly observed that urban males are more aggressive than rural males. However, gaining a holistic understanding of the effects of urbanization on animal behavior requires studying individuals of all sexes and age classes. Finally, I wanted to understand how differences in territorial behavior observed between urban and rural males constrained their ability to provide paternal care, and how this trade-off effects female behavior and their offspring outcomes. Collectively, this research will give us insight into the physiological mechanisms of habituation to urban habitats, as well as the factors that drive behavioral shifts in urban environments, and the costs associated with these shifts.

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Chapter 1: Hypothalamic-pituitary-adrenal axis regulation and organization in urban and rural song sparrows

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Keywords: urbanization, maternal care, female aggression

Link to published article: https://www.sciencedirect.com/science/article/pii/S0016648021001027

Abstract

Urban habitats present animals with persistent disturbances and acute stressors not present in rural habitats or present at significantly lower levels. Differences in the glucocorticoid stress response could underlie colonization of these novel habitats. Despite urban habitats characterization as more stressful, previous comparisons of urban and rural birds have failed to find consistent differences in baseline and stress induced glucocorticoid levels. Another aspect of glucocorticoid regulation that could underlie an animal's ability to inhabit novel habitats, but has yet to be well examined, is more efficient termination of the glucocorticoid stress response which would allow birds in urban habitats to recover more quickly after a disturbance. The glucocorticoid stress response is terminated by negative feedback achieved primarily through their binding of receptors in the hippocampus and hypothalamus and subsequent decreased synthesis and release from the adrenals. We investigated if male song sparrows (*Melospiza melodia*) in urban habitats show more efficient termination of the glucocorticoid stress response than their rural counterparts using two approaches. First, we measured glucocorticoid receptor, mineralocorticoid receptor and 11β-HSD2 (an enzyme that inactivates corticosterone) mRNA expression in negative feedback targets of the brain (the hippocampus and hypothalamus) as a proxy measure of sensitivity to negative feedback. Second, we measured plasma corticosterone levels after standardized restraint and again following a challenge with the synthetic glucocorticoid, dexamethasone, as a means of assessing how quickly birds decreased glucocorticoid synthesis and release. Urban and rural song sparrows had similar reductions in corticosterone following the dexamethasone challenge, suggesting that they do not differ in how quickly they decrease glucocorticoid synthesis and release. Further, though there were no differences in the hypothalamus, urban birds had lower glucocorticoid receptor and 11β-HSD2

mRNA expression in the hippocampus. Thus, urban and rural song sparrows display similar termination of the glucocorticoid stress response even though urban birds have decreased hippocampal glucocorticoid receptor and 11β-HSD2 abundance.

Introduction

Urban habitats present animals with novel and unpredictable stimuli, which are inferred to be stressors, that are either not present or far less frequent in their native habitats (Lowry et al., 2013). These challenges include human disturbance, artificial light at night, increased ambient noise, habitat fragmentation and degradation, and altered anthropogenic predator communities (Injaian et al., 2018; Kleist et al., 2018; Loss et al., 2013; McKinney, 2006; Navara and Nelson, 2007; Rosenberg et al., 2019). Species differ in their responses to such urban stressors, with some species, termed urban avoiders, remaining in their native rural habitats and other species, termed urban exploiters, almost exclusively settling in urban areas (Blair, 1996; Bonier, 2012; McKinney, 2002; Shochat et al., 2006). Species between these two extremes are termed urban adapters and show among them individual variation in their response to urbanization, such that some individuals within a species colonize urban habitats while others remain in native habitats. Making comparisons across species that differ in their response to urbanization, and also among individuals within urban adapter species, can identify the characteristics that allow some animals to live in urban areas despite the additional stressors present. Within species comparisons of urban adapters are of particular importance because they control for life history traits such as sociality, mating system, and migration that may themselves impact an individual's ability to respond to the challenges of urban life (Bonier, 2012).

One endocrine mechanism that could allow animals in urban habitats to persist in the face of novel disturbances is the glucocorticoid stress response. In response to stressors, animals secrete catecholamines from the adrenal medulla to initiate physiological and behavioral reactions, and glucocorticoids from the adrenal cortex that act more slowly and facilitate recovery (Romero et al., 2009; Sapolsky et al., 2000; Siegel, 1980). While the glucocorticoid stress response is essential to coping with disturbances, repeated exposure to novel and unpredictable stimuli and the resulting increases in glucocorticoids can cause stress related disease, oxidative stress, and neural damage (Dickens et al., 2009; Lin et al., 2004; Lynn et al., 2010; McEwen, 2006; Sapolsky et al., 2000). It has been hypothesized that animals in urban habitats characterized by more frequent disturbances could mitigate the damages of repeated glucocorticoid exposure by maintaining lower baseline levels or releasing less glucocorticoid during a stress response (Bonier, 2012). Indeed, several prior studies in songbirds have tested the hypothesis that individuals in urban habitats have lower baseline corticosterone (Cort, the predominant avian glucocorticoid) or dampened release of Cort in response to a standardized stressor (In the focal population: Foltz et al., 2015a; See Review: Bonier, 2012). Results from these comparisons of urban and rural individuals have been mixed, though, and there are no consistent patterns of difference in the endocrine profiles of urban and rural animals (see reviews in Vertebrates: Renthlei et al., 2017; Birds: Bonier, 2012). However, recent research has demonstrated that individuals also vary in the efficiency with which they terminate the glucocorticoid stress response (Liu et al., 1997; MacDougall-Shackleton et al., 2013; Romero et al., 2009; Zimmer et al., 2019). More efficient termination of the glucocorticoid stress response could underlie faster behavioral and physiological recovery and reduce the duration of exposure to glucocorticoids. This raises a new hypothesis; individuals that live in habitats with frequent stressors, such as urban habitats, could benefit from more efficiently terminating the stress response.

Termination of the glucocorticoid stress response could be mediated by greater expression of the receptors that facilitate negative feedback, or by reduced expression of enzymes that metabolize glucocorticoids in brain regions involved in negative feedback. Glucocorticoid secretion is primarily regulated by the hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine cascade that is highly conserved across vertebrate species (McEwen, 2001). This cascade is triggered by internal or external stimuli that induce the hypothalamus to release corticotropin-releasing hormone, which signals the pituitary to release adrenocorticotropic hormone, which in turn stimulates the adrenal cortex to up-regulate the production of glucocorticoids (McEwen, 2001; Romero et al., 2009; Sapolsky et al., 2000). Glucocorticoid secretion is terminated when glucocorticoids bind low affinity glucocorticoid receptors (GRs) within the hypothalamus and hippocampus, causing negative feedback and reduced glucocorticoid synthesis (Dickens et al., 2009; Kadhim et al., 2019; McEwen, 2001; Jacobson and Sapolsky, 1991; Sapolsky et al., 2000; Vandenborne et al., 2005; Zimmer and Spencer, 2014). Greater expression of low affinity GRs relative to higher affinity mineralocorticoid receptors (MRs) is associated with stronger negative feedback on the HPA axis and lower behavioral stress reactivity (De Kloet et al., 1993; Harris et al., 2013; Liu et al., 1997; Meaney, 2001; Zimmer and Spencer, 2014). Additionally, the enzyme 11β-hydroxysteroid dehydrogenase Type 2 (11β-HSD2) (McEwen, 2001; Pérez et al., 2020; Rensel et al., 2018; Seckl, 1997) modulates the effectiveness of Cort by converting it into 11- dehydro - corticosterone and cortisone as it enters the cell, before it can bind GRs (Rensel et al., 2018). Increased expression of 11β-HSD2 could buffer tissue from the effects of glucocorticoids by decreasing their local availability (Pérez et al., 2020; Rensel et al., 2018; Seckl, 1997) but is thought to diminish the strength of negative feedback (McEwen, 2001; Pérez et al., 2020; Wada, 2015). Differences in potential for negative feedback on the glucocorticoid stress response can be estimated using mRNA expression of GR and 11β-HSD2 within brain tissue (Rensel et al., 2018; Zimmer and Spencer, 2014). Additionally, functional termination of the stress response can be evaluated by administering a synthetic glucocorticoid, such as dexamethasone (Dex), and measuring the magnitude of decrease in circulating Cort (Carroll et al., 1981; Lattin and Kelly, 2020). Dex, acting primarily at the pituitary and periphery, selectively binds GR's, so the magnitude of decrease in endogenous Cort reflects how quickly and dramatically an individual terminates glucocorticoid synthesis (De Kloet, 1997; MacDougall-Shackleton et al., 2013; McEwen, 2001). Very few studies have considered how urbanization affects the termination of the glucocorticoid stress response (though see Fokidis et al., 2009). Examining the efficiency of the mechanisms that return animals to their physiological baseline could provide new insights into how variation in HPA axis function supports persistence in novel habitats including urban environments.

Song sparrows (*Melospiza melodia*) are a common North American songbird that establish seasonal breeding territories in both rural and urban habitats. As urban adapters, song sparrows have been the focus of urbanization research for years and express both behavioral and physiological adjustments to urban environments, making them an excellent model for the current study. Past research on the focal populations have had mixed results when investigating the glucocorticoid stress response, with urban and rural birds expressing yearly variation in baseline and stress induced levels. (Beck et al., 2018; Davies et al., 2018; Foltz et al., 2015a). Exploring the endocrine mechanisms of negative feedback in urban and rural song sparrows will add insight into the physiological acclimations that may underlie the consistent behavioral differences observed between urban and rural dwelling animals.

In the current study we tested the hypothesis that individuals in urban habitats terminate the glucocorticoid stress response more efficiently than their rural counterparts, using song sparrows as a study system. We asked if urban and rural birds differed in potential for negative feedback by quantifying the expression of MR, GR, and 11β-HSD2 and we asked if the functional termination of glucocorticoid synthesis differed using a dexamethasone challenge. We predicted that urban and rural birds would have similar relative mRNA expression of MR. We also predicted that urban birds would have greater expression of GR, which would facilitate negative feedback on the HPA axis, and reduced expression of 11β-HSD2, which would increase local availability of glucocorticoids to enhance negative feedback. Additionally, in a second experiment, we predicted that urban birds would show a greater reduction in circulating Cort in response to Dex injection than rural birds.

Methods

1.1. Study population

Male song sparrows in breeding condition were captured in the wild from 3 urban and 3 rural sites near Blacksburg, VA. These sites have been previously characterized for their levels of urbanization using a technique validated by Seress and colleagues (See Davies and Sewall, 2016 and Davies et al., 2018 for details on site selection and characteristic; Seress et al., 2014). All birds were captured via mist nets using 1 of 16 conspecific songs played at the center of previously mapped territories. All captures occurred between 0500 and 1115 hours. All procedures were preapproved by Virginia Tech's Institutional Animal Care and Use Committee and were conducted under current scientific collecting permits.

1.2.: Experiment 1: qPCR quantification of targets for negative feedback

In 2016 we collected territory holding males (urban n = 16, rural n= 10) from the 6 sites during the breeding season (May 4th – May 25th). We captured males within an average of 10.2 minutes of playback (range: 1.2 - 22.22 minutes). We immediately anesthetized each male with isoflurane, euthanized them, and removed their brains which were flash frozen on dry ice and stored at −80°C until qPCR was performed. We sectioned half of the brain in 40 μm sections in a cryostat set to -20ºC. Using a scalpel, we micro-dissected out the hypothalamus and hippocampus with the central fissure and anterior commissure as anatomical landmarks, found by referencing a passerine bird brain atlas (Nixdorf-Bergweiler and Bischof, 2007). We extracted total RNA from each brain region using a commercially available kit following the manufacturer's instructions (Total RNA Purification Micro Kit, Norgen Biotek, Canada, Cat. No. 35300) and we included the DNase-1 digestion step (RNase-Free DNase-1 Kit, Norgen Biotek, Canada, Cat. No. 25710). To assess RNA quantity and purity we used a nano-spectrometer (Nanophotometer Pearl, Implen, USA) and used a Bio-Analyzer 2100 (Agilent Technologies, USA, Cat. No. G2939BA) to assess RNA integrity. We reverse transcribed 100ng of total RNA to cDNA using a commercially available kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Cat. No. 4368813) and diluted with RNase-free water to a final concentration of 1ng/ul. Following the manufacturer's instructions, the thermocycler conditions were 25 °C for 10 minutes to start, then 37 °C for 120 minutes, 85 °C for 5 minutes, and then the thermocycler held the product at 4°C until it was taken out. A song sparrow genome has not been published, so we used the zebra finch (*Taeniopygia guttata*) genome for primer design. We designed primers for GR, MR, 11β-HSD2, and the reference gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, which did not differ between treatment groups) using Primer Express v.3 (Applied Biosystems, USA, Cat. No. 4363991), and the oligonucleotides were synthesized by Integrated DNA Technologies, USA (see Table 1 for Accession Numbers and primer sequences). The Ct values for GAPDH were cross-calibrated against values of other reference genes including beta-actin and 18S rRNA and determined to be the most stable across all sample types.

Primer specificity was confirmed before experiments via Primer-BLAST (NCBI; default search parameters and taxid:59729) of each primer pair against reference genomic sequences for the zebra finch. No additional target templates were identified. Specificity of starting template (ie., lack of genomic DNA carryover) was confirmed via lack of PCR amplification for negative control samples that were generated from reverse transcription reactions that did not contain reverse transcriptase. We performed the qPCR reactions in MicroAmp Fast Optical 96-Well Reaction Plates (Applied Biosystems, USA, Cat. No. 4346906) with MicroAmp Optical Adhesive Film (Applied Buosystems, USA, Cat. No. 4311971) using Fast SYBR Green Master Mix (Applied Biosystems, USA, Cat. No. 4385612). We ran all samples in duplicate, with all samples for a specific gene of interest within a specific brain region run on a single plate (six plates total). For instance, plate 1 being GR in the hippocampus, plate 2 being GR in the hypothalamus etc. Each well contained 3μl cDNA, 0.25μl of 5μM forward primer, 0.25μl of 5μM reverse primer, 5μl Fast SYBR green, and 1.5μl RNase-free water. We calculated standard curves to assess amplification efficiency (90-110%). These were validated to be similar between target genes and the reference gene via standard curves generated from serial dilutions of cDNAs from multiple samples (90 to 110%; 100% for reference gene). We ran all plates on an Applied

Biosystems 7500 Fast Real-Time PCR System (SeqGen Inc., USA) with the following cycling parameters: 95ºC for 20 seconds, followed by 40 cycles of 95ºC for 3 seconds and 60ºC for 30 seconds. Post-PCR, amplicon specificity was confirmed via melting curve analysis where a single peak representing the amplified product for each reaction was observed at the correct melting temperature (95 °C for 15 seconds 60 °C for 1 minute, 95 °C for 15 seconds and 60 °C for 15 seconds).

1.3.Experiment 2: Dexamethasone challenge

In 2019, using a separate cohort of birds, we compared the efficiency with which urban and rural males terminated the glucocorticoid stress response using a Dex challenge in the pre-breeding season (March 23rd –April 13th). We targeted territories that had been established in the previous breeding season (2017 and 2018). To confirm a male was on the territory, we played 1 of 16 recordings of conspecific songs for 5-20 seconds within the boundaries of a known territory. When we confirmed that a male was present, we placed a mist net in an observed flight path and played pre-recorded conspecific song for up to 68 minutes. We captured males within 24.4 minutes on average (range of 1 - 68 minutes) and immediately removed them from the net and placed them in a breathable cloth bag to induce a glucocorticoid stress response. To determine stressor-induced Cort levels, we collected a 70 µl blood sample 30 minutes after capture via puncture of the brachial veins using a 26-gauge needle. We randomly assigned every other bird captured to receive an injection of either Dex or vehicle (phosphate buffered saline, PBS) except for two days of the experiment, when all birds received injections of Dex (Dex: urban n = 13, rural n = 14; saline: urban n = 7, rural n = 8). Birds assigned to the Dex condition were given a 100 µl intramuscular injection of 1mg/kg Dex (crystalline Dex, Sigma Aldrich, Cat.

No. D1756) dissolved in ethanol, brought to volume with autoclaved PBS (ethanol was <5% of the final volume; Holberton et al., 2006), while birds assigned to the saline condition were given an intramuscular injection of saline (autoclaved PBS diluted with ethanol; ethanol was <5% of the final volume). This Dex dose has been shown to effectively induce termination of the glucocorticoid stress response in several songbirds, including song sparrows (Bauer et al., 2016; MacDougall-Shackleton et al., 2013; Schmidt et al., 2012). After injection, we placed all birds in covered cages with seed and water for 1 hour (Schmidt et al., 2012) and then collected a second 70µl blood sample to assess the effect of Dex or saline injection on glucocorticoid levels (Lattin and Kelly, 2020; MacDougall-Shackleton et al., 2013). We stored all blood samples on ice immediately after collection and centrifuged them later the same day. We stored plasma at -20°C until assayed for Cort.

1.3.1. Cort assay

We quantified Cort levels using a commercially available enzyme-linked immunosorbent assay (ELISA; Lot No: 1201810, Enzo Life Sciences, Inc, Farmingdale, NY) following the manufacturer's instructions. Previous research has validated the assay for song sparrows, and we assessed cross-reactivity with Dex (see below; Davies and Sewall, 2016; Davies et al., 2018). Briefly, we diluted samples 1:40 and added, 1% steroid displacement reagent. We assayed samples in duplicate, with all samples from a given bird assigned to the same plate and all birds randomly assigned to one of two plates. Intra-assay variation was 11.14% and assay sensitivity was 27pg/ml. Inter-assay variation was controlled for by including plate number as a random effect in the final model. To evaluate cross-reactivity with Dex for this brand of ELISA we ran samples of song sparrow serum spiked to 2, 20, 50, 100 and 200 ng/mL Dex. We found low cross-reactivity at 50 ng and below, but it increased at higher concentration (see Supplementary Materials). Because of this and recommendations from recent publications (Lattin and Kelly, 2020) we will be referring to relative amounts (relative change from stress induced Cort to 60 minutes post Dex injection) of Cort and not absolute values after injection.

1.4.Statistical analysis

The statistical analyses presented in the manuscript were conducted using SPSS (v.25) and R (v. 3.6.1: R Core Team, 2019). For qPCR data, we calculated relative expression of each gene of interest using the ΔΔCt method, i.e. $2^{-\Delta\Delta Ct}$ (ΔCt = target gene Ct – GAPDH Ct, ΔΔCt = ΔCt – calibrator ΔCt where the calibrator is the mean ΔCt of rural birds)(Schmittgen and Livak, 2008). We analyzed relative expression of each target gene (MR, GR,11β-HSD2) in each brain region (hippocampus, hypothalamus) in separate generalized linear models with data fitted to a gamma error distribution and habitat type entered as a fixed factor in each model. The deviance residuals from these models were examined for normality. We explored all significant effects further using Sidak *post hoc* tests, and calculated Cohen's d effect size for all significant *post hoc* tests.

We assessed termination of the stress response by calculating the relative change in Cort concentration from stress induced levels (((30minute sample – 90minute sample)/30minute) x100%) following recommended methods in Lattin and Kelly (2020). We entered the relative change in Cort as the dependent variable in the linear mixed effect model with habitat type, injection type, and the interaction between them entered as fixed factors. We entered time of capture as a co-variate to account for circadian changes in Cort concentration and negative feedback. We assessed the effect of habitat type on stressor - induced (30 minute) Cort by entering 30 minute Cort as the dependent variable in a second linear mixed effect model with habitat type as the fixed factor. We used time to capture (also referred to as playback exposure time) and day of year as co-variates. Plate number was entered as a random effect in both these models to account for inter-assay variation. Residuals were normally distributed (Shapiro-Wilk, $p > 0.05$). We used an alpha value of 0.05 as the threshold for statistical significance. All figures show mean +/- 1 standard error of the mean.

Results

1.5.Experiment 1: qPCR quantification of targets of negative feedback

We found no difference between urban and rural song sparrow's hypothalamic mRNA expression of GR ($\chi^2_{1,24} = 1.473$, $p = 0.237$), MR ($\chi^2_{1,24} = 0.778$, $p = 0.386$) or 11β-HSD2 ($\chi^2_{1,24} = 0.820$, $p = 0.374$) (Figure 1). In the hippocampus however, urban song sparrows had lower hippocampal mRNA levels of GR ($\chi^2_{1,24} = 6.953$, $p = 0.014$, $d = 1.056$) and 11β-HSD2 ($\chi^2_{1,24} = 10.474$, $p = 0.004$, $d = 1.171$) relative to rural song sparrows (Figure 1). Hippocampal MR mRNA expression did not differ between habitats ($\chi^2_{1,24} = 0.367$, $p = 0.550$).

1.6.Experiment 2: Dexamethasone Challenge

Injection with Dex significantly decreased Cort from stress induced levels compared to saline injection ($t_{1,37} = 3.12$, p=0.004, d=1.25). Thus, our Dex injection successfully induced negative feedback. However, the interaction between habitat type and injection type was not significant ($t_{1,37} = 0.53$, p=0.60; Figure 2), indicating that there was no difference in how urban and rural birds responded to Dex. Independent of treatment group, rural birds had significantly higher stressor-induced Cort (pre-injection) compared to urban ($t_{38,1}$= -2.24, p=0.031, d=0.78; Figure 3).

Discussion

Urban habitats are often considered more stressful than native habitats because they present individuals with unpredictable and novel stimuli (Birnie-Gauvin et al., 2016; McKinney, 2002) and the focus of many urbanization studies has been understanding how animals cope with these potential stressors. In the current study, we hypothesized that more efficient termination of the glucocorticoid stress response might underlie the ability of some individuals that colonize and persist in novel urban habitats. To test this hypothesis, we compared (1) the expression of mRNA for receptors and enzymes (MR, GR, and 11β -HSD2) that mediate negative feedback within the hypothalamus and hippocampus and (2) the magnitude of relative decrease in circulating glucocorticoids after injection with the synthetic glucocorticoid Dex (aka a Dex challenge), between urban and rural male song sparrows. We predicted that urban birds would have greater mRNA expression of GR, and lower expression of 11β-HSD2, which would facilitate negative feedback of the HPA axis. Additionally, we predicted that urban birds would have a stronger response to the Dex challenge than rural birds and reduce circulating Cort to a greater degree following injection.

Counter to our predictions, urbanization is not associated with any detectable differences in the termination of the glucocorticoid stress response as measured by the Dex challenge. Treatment with Dex significantly lowered circulating levels of Cort relative to saline injection, but urban and rural song sparrows did not differ in how much their circulating Cort was reduced in response to Dex (Figure 2). Urban birds had significantly lower stress induced Cort following a standardized stressor as compared to urban (Figure 3) and urban birds expressed a similar negative feedback to rural birds suggesting that overall urban birds are minimizing their HPA activation in response to stressors. A muted glucocorticoid stress response could protect individuals from the negative effects of chronic exposure to glucocorticoids. Additionally, this could be a physiological acclimation to a novel habitat, where disturbances and encounters with acute stressors occur often. However, previous research on this population has shown variation across years in both stress-induced and baseline levels of Cort in adult male song sparrows (Foltz et al., 2015a). Foltz et al. found that in some years males from urban habitats would express increased baseline and/or stress induced levels of Cort compared to rural, while in other years the patterns would be reversed or nonexistent. These conflicting results over the glucocorticoid stress response of urban birds and a lack of functional differences in negative feedback mechanisms highlight the complex interactions occurring within urban habitats and the variability of the glucocorticoid stress response. It is also important to note that a Dex challenge primarily reflects negative feedback mechanisms occurring at the pituitary and the periphery and the termination of glucocorticoid synthesis and mechanisms of negative feedback are not completely synonymous (De Kloet, 1997; Lattin and Kelly, 2020). Future studies that bring animals from urban and rural habitats into captivity could examine urban males ability to release Cort through an ACTH challenge, and would be able to further examine negative feedback by administering Dex directly to the brain (Pérez et al., 2020).

Though we found no evidence of functional differences in the termination of glucocorticoid synthesis, we found habitat differences in mechanisms implicated in mediating negative feedback of the HPA axis. We found, contrary to predictions, lower relative expression for both GR and 11β-HSD2 in the hippocampus of urban birds (Figure 1). Lower hippocampal GR expression is generally associated with reduced negative feedback on the glucocorticoid stress response, which would suggest urban males have less capacity for negative feedback on the HPA axis (Liu et al., 1997; McEwen, 2001; Dickens et al., 2009; Zimmer and Spencer, 2014). The finding that urban males had reduced hippocampal 11β-HSD2 is less straight forward. 11β-HSD2 metabolizes glucocorticoids as they enter the cell, inactivating them, so decreased abundance would increase available glucocorticoids in the hippocampus. Without the reduction in hippocampal GR, this could increase the strength of negative feedback (Pérez, et al., 2020), but a reduction in both could cancel out any negative feedback effects. However, another hypothesized function of 11β-HSD2 is protection against the damaging effects of elevated glucocorticoids (Oppermann et al., 1997; Pérez et al., 2020; Rensel et al., 2018). Therefore, urban males with lower 11β-HSD2 may be at risk of greater damage from glucocorticoids in the hippocampus. We found no habitat differences in the expression of receptors or enzymes in the hypothalamus (Figure 1). Collectively, these results do not support the hypothesis that urban male song sparrows terminate the glucocorticoid stress response more efficiently and even suggest that urban males have reduced capacity for negative feedback. By evaluating both the function of the HPA axis using a Dex challenge and the key brain mechanism by which we understand negative feedback to act, we find that negative feedback on the HPA axis and termination of the glucocorticoid stress response does not facilitate the settlement of urban habitats by song sparrows. However, future studies in species that are more recent colonists of urban areas or across more dramatic habitat gradients should be pursued. Also, it is important to acknowledge that relative mRNA expression is not the same as functional protein levels, and that urban and rural birds could differentiate levels of protein production downstream of mRNA expression. Additionally, we view this as a starting point, and that further studies using immunohistochemistry to look at protein expression and receptor binding as well as enzyme activity assays are required to gain a full understanding of the changes occurring in these brain regions of urban adapters.

Though our findings do not support our hypothesis that faster termination of the glucocorticoid stress response underlies colonization and persistence in novel urban habitats, they do raise a new hypothesis. In previous work, we found no evidence of population genetic structure (Brewer et al., 2020) and though theoretically there could be specific genetic alteration between these groups, we believe they are one continuous interbreeding population. This suggests individuals in our urban populations are experiencing some level of developmental organization or lifelong acclimation to urban habitats. In other systems it has been shown that the expression of GR can be organized by early life experiences and may be decreased by exposure to stressors (Dickens et al., 2009; Liu et al., 1997; McCormick et al., 2005; Zimmer and Spencer, 2014). Urban habitats are well-documented to have more frequent disturbance and song sparrows at our study sites experience more frequent human disturbance and higher ambient noise (Akçay et al., 2020; Foltz et al., 2015a, 2015b). Conversely, though urban habitats are characterized as being more stressful, this characterization may be premature. Many urban habitats also provide animals with additional resources (anthropogenic sources of food, water, and nesting substrates) and protection from predators (Reynolds et al., 2019; Shochat et al., 2010; Soulsbury and White, 2016). Urban habitats differ considerably from more native rural habitats, but they may no longer be stressful to animals that have acclimated to living within them. The glucocorticoid stress response assists with recovery from acute stressors, and its activation may decrease with acclimation to the novel stimuli present in urban habitats.

Overall, the current study provides evidence of brain region specific differences in receptor and enzyme abundance between urban and rural adult male song sparrows, even though there are no consistent differences in levels of circulating hormones. We did not detect differences in the termination of the glucocorticoid stress response using a Dex challenge. We did find that urban males have lower stress induced Cort levels, suggesting urban individuals have a dulled glucocorticoid stress response. However, past studies in our lab (Davies et al., 2018) and others (Foltz et al., 2015a) failed to find consistent differences in baseline or stress induced Cort, casting doubt on this conclusion. Finally, we found differences in the HPA axis associating urbanization with significantly reduced hippocampal GR and 11β -HSD2 abundance. Additional studies are needed to determine if these differences result from developmental programming by urban habitats or lifelong acclimation to urban habitats. Importantly, future work also needs to examine the HPA axis response to stressors other than restraint to determine if animals in urban habitats are habituating selectively to urban stressors and do not, in fact, show altered HPA axis function.

Acknowledgments

We thank B. Cuellar, C. R. Linkous, and K. Pontarelli for assistance in the field and I. T. Moore and J. Hernandez for comments on manuscript drafts. This research was supported by the Global Change Center at Virginia Tech and National Science Foundation grant IOS 1652496

Figures and Tables

Table 1

Accession numbers and primer sequences

GOI	Accession No.	Forward Sequence	Reverse Sequence
MR	NM_001076690.1	CGAGCCCTCCGTCAACAC	GGAGTAAGTGCTGGTGAGATAGCA
GR	XM_002192952.4	TCTCCCCTCGTGCACCAT	TGTTCGTAACAGCCTCAGAGCTT
11β -HSD2	XM_030282630.1	GCGAGGACTATGTGGAGGAGAT	TCCACTGCCACCTTCATGAA
GAPDH	XM_030266469.1	GTGGTGCCAAGCGTGTGA	CACGAACATGGGAGCATCAG

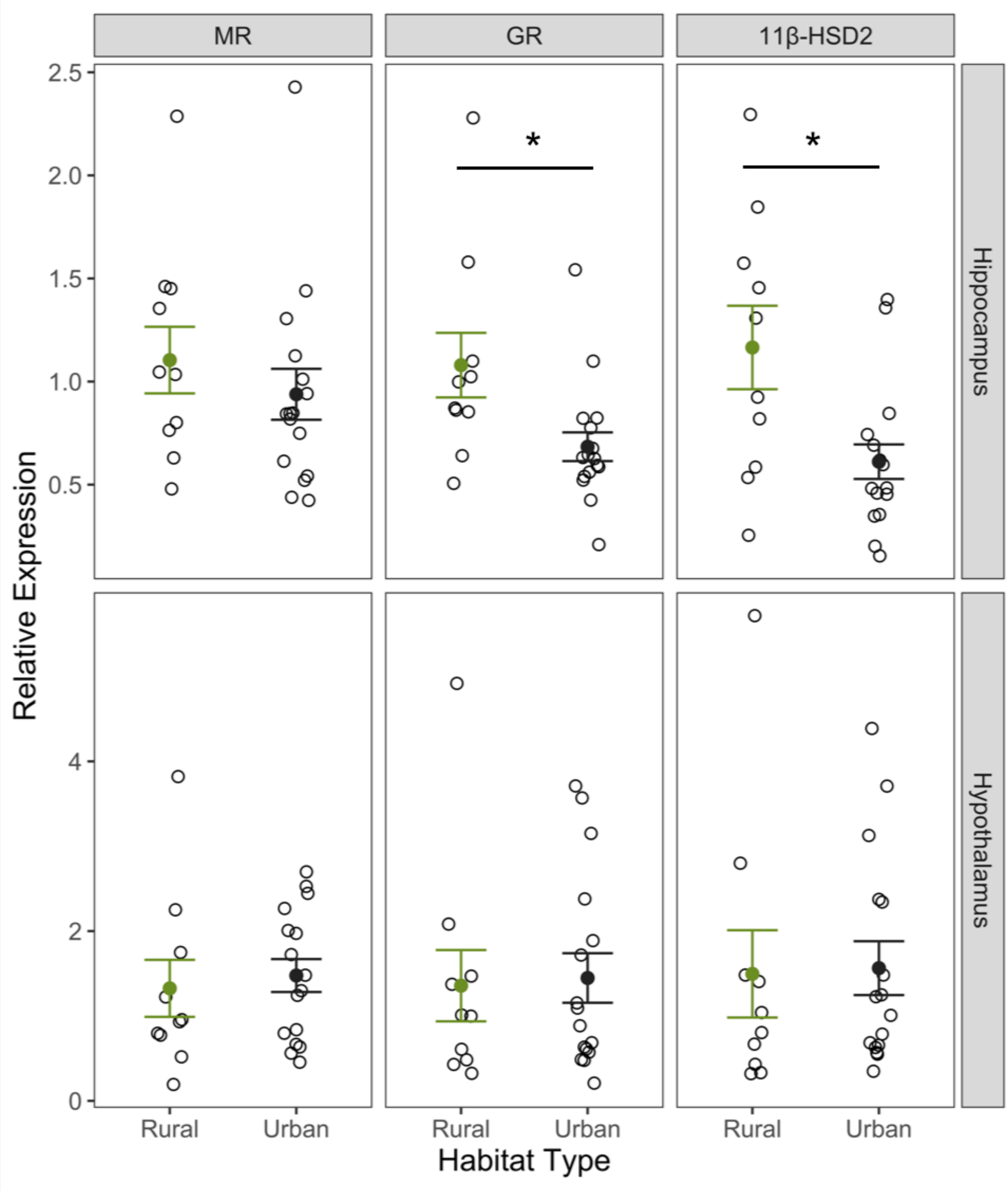

Figure 1: Relative expression of hypothalamic and hippocampal mRNA levels of glucocorticoid receptors (GR), mineralocorticoid receptors (MR), and 11β-hydroxysteroid dehydrogenase (11β-HSD2) between urban and rural song sparrows. Open circles are individuals collected in 2016,

with means ± 1 standard error of the mean and data were normalized to GAPDH. Song sparrows, regardless of habitat, have similar hypothalamic mRNA levels of GR, MR, and 11β-HSD2. Urban male song sparrows, relative to rural males, have lower hippocampal mRNA levels of GR and 11β-HSD2 (* = $p < 0.05$). Hippocampal MR mRNA expression is similar in all birds regardless of habitat

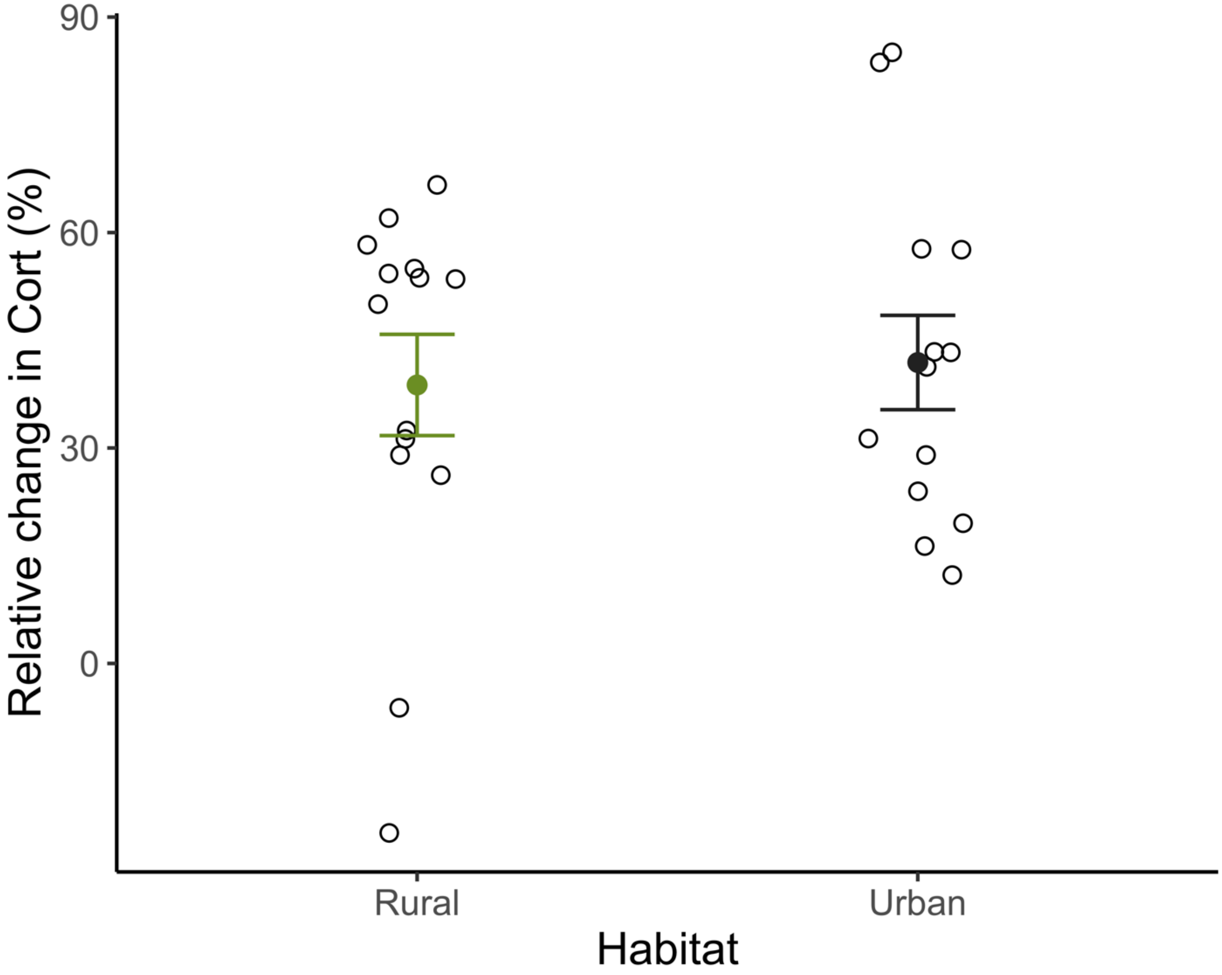

Figure 2: Relative reduction of circulating Cort (ng/ml) following Dex injection in 2019 urban and rural male song sparrows. Open circles are individuals, with means ± 1 standard error of the mean. Birds had comparable relative reductions in Cort in response to Dex regardless of habitat

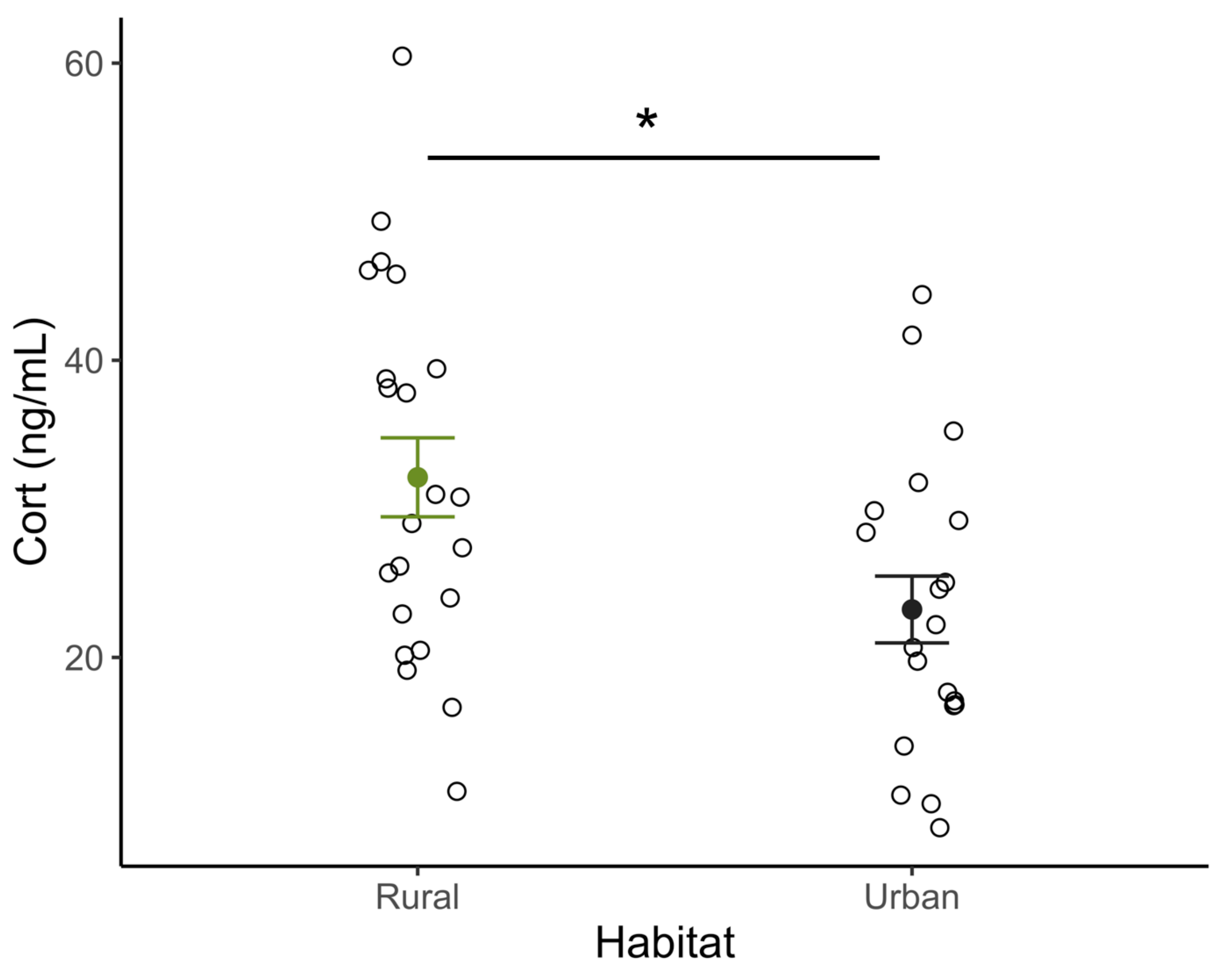

Figure 3: Stress induced Cort levels following 30 minutes of restraint in urban and rural male song sparrows. Open circles are individuals, with means ± 1 standard error of the mean. In 2019 rural song sparrows (n = 22) had higher stress induced Cort compared to urban (n = 20) pre-injection injection and independent of treatment group.

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Appendix A: Chapter 1 Supplementary Materials

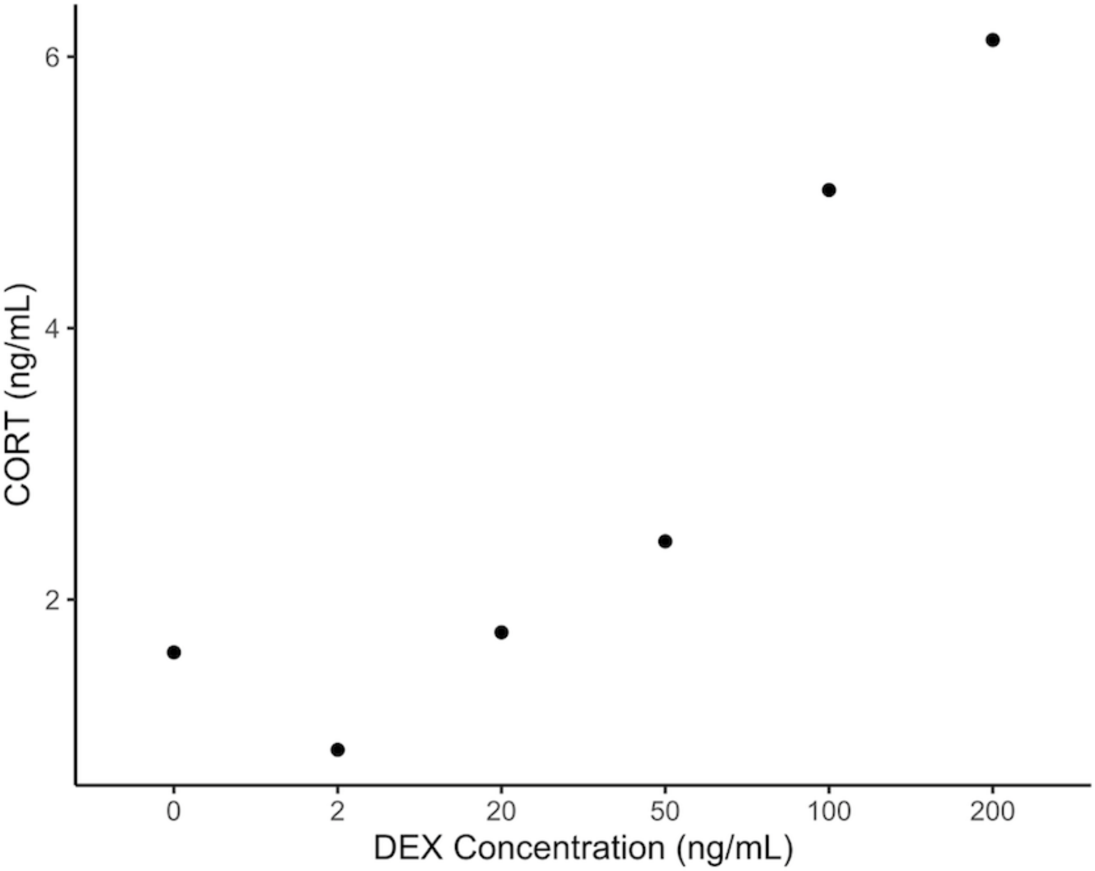

Figure A1. Cross reactivity test for Enzo Life Sciences Cort ELISA. Stripped song sparrow plasma (DEX Concentration 0 ng/mL) was spiked to 2, 20, 50 100 and 200 ng/mL DEX. Samples were run in duplicate with the unstripped plasma (not shown, mean = 14.19 ng/mL Cort).

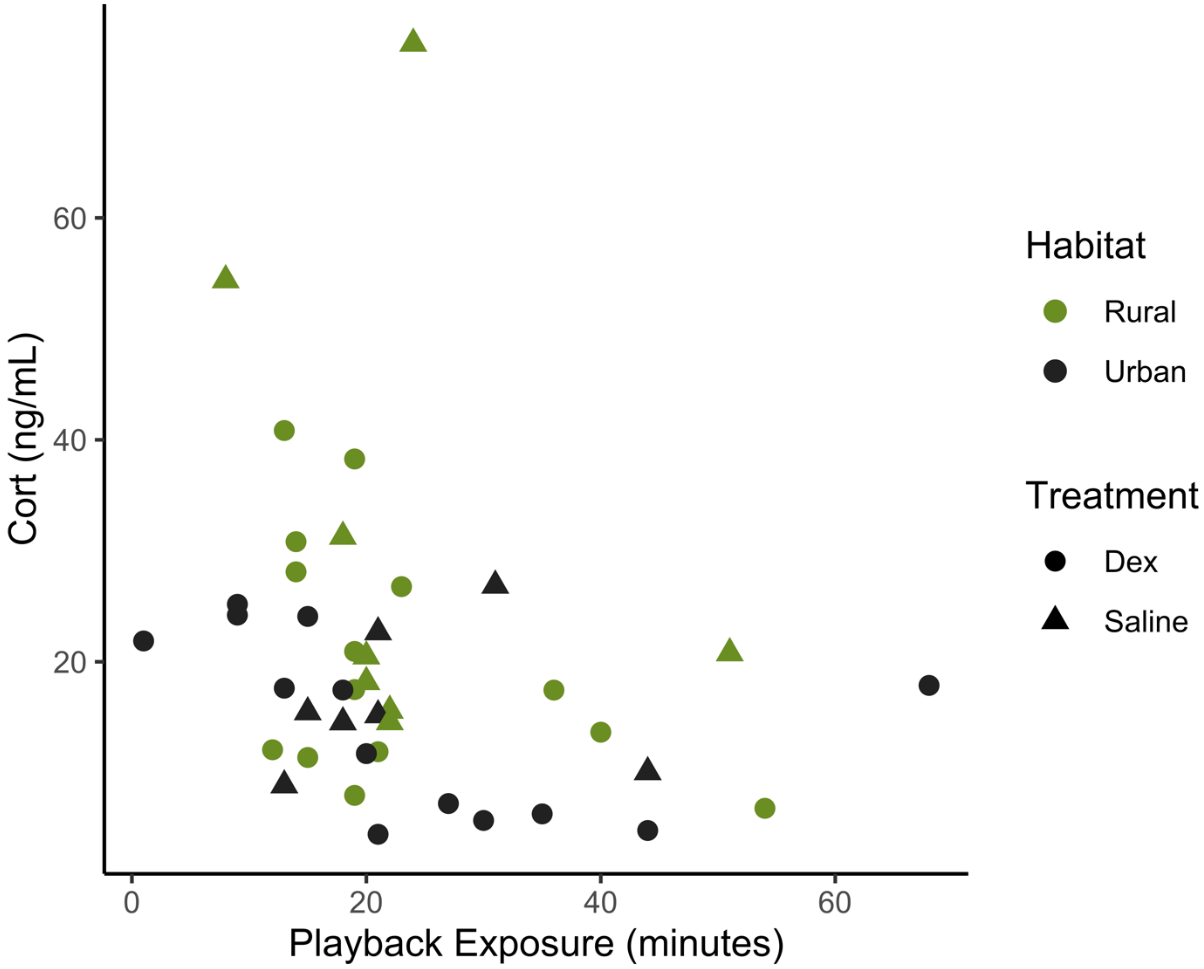

Figure A2: The relationship between Recovery (90 minute post capture) Cort levels and playback exposure time (time to capture). There was no relationship between 90 minute Cort levels and capture time when treatment group (saline or Dex injection) is accounted for.

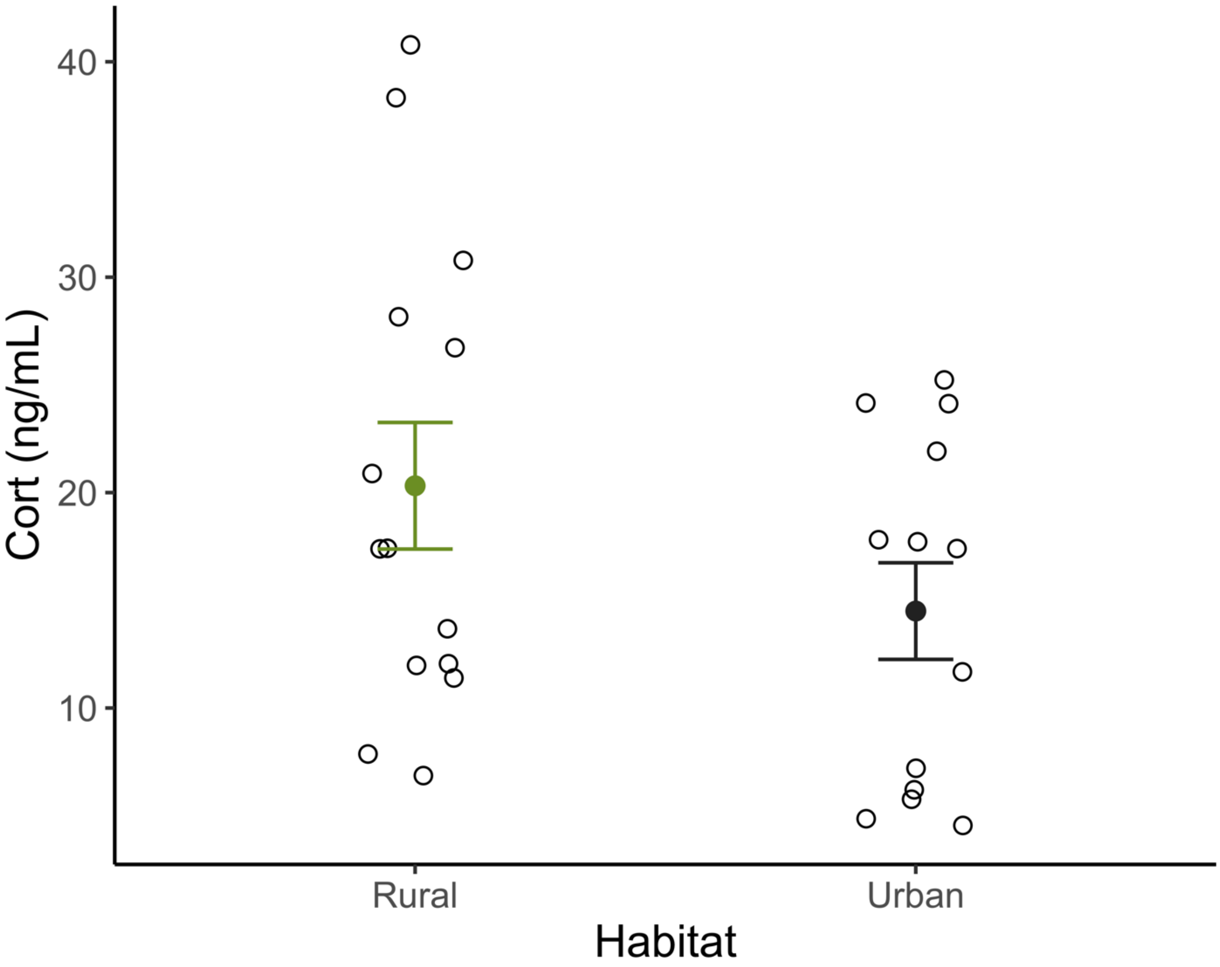

Figure A3: Rural song sparrows (n = 14) had similar Cort levels (mean ± SEM= 20.32 ± 2.94) as urban (n = 13; mean ± SEM =14.50 ± 2.24), 1-hour post injection with Dex. Open circles are individuals, with means ± 1 standard error of the mean.

Chapter 2: What about females? Urban female song sparrows elevate aggressive signaling compared to rural.

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Keywords: urbanization, maternal care, female aggression

Abstract

The costs and benefits of breeding behaviors are influenced by environmental conditions, and habitat variation can shift the degree to which behaviors are expressed. Novel urban habitats have been shown to differ significantly in disturbances such as noise, light at night, and human presence, as well as resource availability, compared to rural habitats. Perhaps because of these environmental differences, urban males of several species are consistently more aggressive than rural males, raising the hypothesis that greater territorial aggression is beneficial in urban habitats. Though often ignored, female songbirds of many species also perform aggressive territorial behaviors towards conspecifics during the breeding season. For socially monogamous songbirds, this aggression functions to ensure partner fidelity and secure resources for reproduction. Studies of the effects of urbanization on songbird behavior have yet to determine if urban females also express greater territorial aggression. Importantly, energetically demanding behaviors such as territoriality and parental care should constrain one another, leading to behavioral trade-offs during the breeding season. Though territorial aggression and parental care are inversely related in males of several species of songbird, this relationship is understudied in female songbirds, particularly those facing environmental change such as urbanization. In this study, we compared aggressive signaling and a measure of parental care (maternal nest visitation rates) between female song sparrows (*Melospiza melodia*), living in urban and rural habitats. We hypothesized that female aggressive signaling would be higher in urban environments compared to rural, and negatively correlated with maternal visitation rates. We found that urban females, like males, expressed increased aggressive signaling compared to rural. However, female aggressive signaling was not related to our measure of maternal care, suggesting females aren't facing a trade-off between these two behaviors. Collectively, our results are consistent with the

hypothesis that urban habitats promote territorial aggression in female song sparrows. As urbanization continues to spread, understanding the behavioral changes animals employ in urban environments requires studying individuals of different sexes and age classes, and will help us understand how some species are able to cope with human induced rapid environmental change.

Introduction

Behavioral shifts are often the first and fastest means by which animals acclimate to environmental changes, including human-induced rapid environmental change (Sih 2013; Sih et al. 2016). Indeed, animals in urban habitats express reliably different behaviors than their rural counterparts (see reviews: Lowry et al. 2013; Renthlei et al. 2017), including decreased neophobia (Battle et al. 2016; Jarjour et al. 2020; Miranda 2013), increased risk-taking behavior (Grunst et al. 2019), conspecific aggression, and boldness (Evans et al. 2010; Foltz et al. 2015). Specifically, urban male songbirds from multiple territorial species approach humans and sham predators more closely (Evans et al. 2010; Fossett and Hyman 2021; Myers et al. 2016) and are more aggressive in response to conspecific models and song playbacks during simulated territorial intrusions (Fokidis et al. 2011; Hardman and Dalesman 2018; Ripmeester et al. 2010; Scales et al. 2011) compared to rural males.

The habitat in which individuals choose to breed has been shown to greatly modulate aggressive and territorial behaviors (Gunnarsson et al. 2005; Holtmann et al. 2017). The benefits of territorial aggression depend upon how easily resources are defended (Emlen and Oring 1977). In urban habitats, frequent disturbance and habitat fragmentation alter resource distribution and availability (Isaksson 2018; Farwell and Marzluff 2013), which could make increased territorial aggression beneficial. Even low-density urbanization transforms landscapes, fragments habitats into discrete blocks, and is associated with increased territorial aggression in male songbirds of several species (Davies and Sewall 2016; Evans et al. 2010; Ewers and Didham 2006; Foltz et al. 2015; Gomes et al. 2011; Hagan et al. 1996; McKinney 2002; Myers et al. 2016; Saunders et al. 1991). However, most studies examining the effects of urbanization on songbird behavior have focused on males. There are many good reasons for this; males are often more conspicuous and easier to find, and they express a variety of easily measurable behaviors. Male songbirds of many species maintain seasonal breeding territories that they defended vigorously and compete with other males over mates and reproductive resources (Catchpole and Slater 2003; Krippel et al. 2017). However, fully understanding the effects of urbanization on the behavior of wild songbirds requires studying individuals that are most impacted by changing environmental conditions, which includes females.

Though less frequently studied, female songbirds do defend breeding territories, most notably in the tropics where females are often more territorial than males (Stutchbury and Morton 2001). The frequency and intensity of female territorial behavior decreases with increasing latitude (Catchpole and Slater 2003), though many female songbirds in the North Temperate zone still assist their partner in maintaining and defending seasonal territories (Cain and Ketterson 2012; Clutton-Brock and Vincent 1991; Elekonich 2000; Griffith et al. 2002; Wingfield 1994). Despite the assumption that females do not need to be territorial in species in which males express heightened territoriality, there are circumstances when it may be advantageous for females to show increased territorial aggression. Female aggression functions to maintain the social partner bond and paternal investment through mate guarding (Cézilly et al. 2000; Marzluff and Balda 1988; Sandell and Smith 1997), and to defend the nest and offspring from conspecifics and predators (Heinsohn et al. 2005; Prosen et al. 2004; Reichard and Boesch 2003). Additionally, females compete for nesting sites, including cavities and territories with substrates for open cup nesters (Cristol and Johnsen 1994; Heinsohn et al. 2005; Pärn et al. 2007; Prosen et al. 2004; Wischhoff et al. 2018). Indeed, recent research has highlighted the importance of female-female competition in maintaining fitness during the breeding season (Cain and Ketterson 2012; Rosvall 2008; Rosvall 2011a; Rosvall 2011b; Rosvall 2013a; Thys et al. 2017). Just as with males, females may also increase their fitness by engaging in territorial aggression in habitats in which resources are scarce or easy to defend, perhaps including urban areas in which nesting substrates and resources may be sequestered into distinct, fragmented segments (Heinsohn et al. 2005; Prosen et al. 2004; Wu et al. 2019). The impact of urbanization on female territorial aggression is understudied (though see Miranda 2014), yet females have higher investment in offspring than males, making resource defense at least as important to female fitness as it is to male fitness (Sandell and Smith 1997; Trivers 1972).

Territoriality and aggressive behaviors require time and energy and increased territorial aggression can generate trade-offs with other reproductive behaviors such a parental care (Bateman 1948; Clutton-Brock and Vincent, 1991; Trivers 1972; Rosvall 2009). In several species of songbirds, more aggressive individuals invest in gaining the best territories and attracting the most mates but provide less parental care (Johnson and Burley 1998; Møller 1991; Sheldon 1994). For example, male songbirds can maximize fitness by decreasing paternal care and increasing extra pair mating or, alternatively, by investing in paternal care to increase offspring condition and survival at the expense of extra pair mating (Arcese 1989; Griffith et al. 2002; Møller, 2000). Historically, research on trade-offs has focused on male songbirds, but recent studies have demonstrated that breeding females also face this trade-off between aggression and parental care (Cain and Ketterson 2013; Rosvall 2008; Rosvall 2009; Rosvall 2013a; Rosvall 2013b; Sheldon 1994; Wischhoff et al. 2018). The trade-off may, in fact, be more significant for females because reproduction is more energetically costly for females in the north temperate zone compared to males (Goymann and Wingfield 2004; Small and Schoech 2015). Thus, if urban habitats influence aggressive behavior in wild female songbirds, it may also impact maternal care behaviors that may be traded-off as part of a reproductive strategy.

Song sparrows (*Melospiza melodia*) are a common North American songbird that live and breed in both urban and rural habitats. Song sparrows form social partnerships during the breeding season to defend a territory and raise a clutch of young, though both pair members seek extra pair mating (15 - 40% of young not sired by the social male; Keller 1998; unpublished data). Both males and females express territorial defense behaviors (Elekonich 2000; Wingfield and Monk 1992; Wingfield 1994) and previous research has shown that urban male song sparrows express greater territorial aggression than rural males during simulated territorial intrusions (Davies et al. 2016; Evans et al. 2010; Foltz et al. 2015). Female song sparrows respond to conspecific females on their territories by approaching and vocalizing (Elekonich 1997; Elekonich 2000). These behaviors have been associated with attacks of conspecifics by female song sparrows and have been referred to as female aggression, though here we use the term aggressive signaling (Arcese et al. 1988; Elekonich 2000). If being more aggressive is beneficial in urban habitats then, like males, urban female song sparrows should show increased aggressive signaling compared to their rural counterparts. However, an increase in territorial aggression could come at the cost of parental care. Therefore, in the present study, we explored the effects of urbanization on the aggressive signaling and nest visitation rates of female song sparrows. Specifically, following Elekonich 2000 we conducted simulated territorial intrusions by placing a female mount (taxidermy female song sparrow) and playing vocalizations from the center of a territory. We quantified the aggressive signaling of the focal female as average distance of approach, closest approach, duration of response, and duration of vocal response. Based on findings in urban male song sparrows we predicted that urban females would be more likely to respond to the simulated territorial intrusion, rather than fleeing the area or continuing with maternal behaviors (e.g., incubation). Additionally, we expected that urban females would express increased aggressive signaling and that they would respond faster and longer, come closer to the playback, and vocalize more often during the simulated territorial intrusion by a female conspecific. We also investigated the possibility of a trade-off between female aggressive signaling and maternal nest visitation and predicted that this measure of maternal care would be negatively correlated with female aggressive signaling across both urban and rural habitats.

Methods

Adult Capture

We captured female song sparrows from six field sites along an urban and rural gradient in southwestern Virginia (for details on field sites and evaluation of urbanization characteristics see Davies et al. 2018). All individuals are part of a long-term study monitoring the fitness and breeding behavior of urban and rural song sparrows (Davies and Sewall 2016; Davies et al. 2018). We located and banded females during the 2018, 2019, and 2021 breeding seasons (March - July) when leaving or returning to their nest. We captured all birds with mist nets between 0500 and 1115 hours. To avoid nest abandonment, we limited capture attempts to 120 min. and never attempted captures on sequential days. We also avoided catching females during nest building and egg laying. At capture we took morphological measurements and, to monitor parental visitation, we fitted a passive integrated transponder (PIT) tag (2.12x10mm; CYNTAG, Inc; Item#: 601205-2248) to each focal bird's tarsometatarsus following the methods established in Bridge and Bonter 2011. All birds were given a unique combination of color bands and multicolored heat shrink tubing (Bridge and Bonter 2011) to allow visual identification during aggression trials.

Female aggressive signaling

To assess female aggressive signaling across habitat types we performed simulated territorial intrusions on 51 urban females and 44 rural females, following methods in Elekonich and Wingfield; 2000. Specifically, to create playback tracks we recorded aggressive vocalizations from 6 females within the focal population and identified calls characterized by Arcese et al. 1988 and Elekonich 1997 as aggressive by comparing spectrograms of our recordings against those publications. We created 12 unique playback tracks, each 9 min. in duration, and played one of each track on a loop during simulated territorial intrusions. We randomly chose playbacks for each female, except in instances of recorded females or their neighbors. For these we chose a track recorded from a different female. We performed all behavioral trials between April and July of the 2018, 2019, and 2021 breeding seasons. Briefly, we placed a speaker (JBL Micro 2) and the taxidermy female song sparrow mount approximately 10 meters from the focal female's nest but within the pair's territory. For females in 2018 and 2019 (43 urban and 37 rural) we played a randomly selected playback track for 9 minutes. In 2021 (8 urban and 7 rural) we played a randomly selected track for only 6 minutes and therefore include year in all the analyses to account for this difference in playback duration. During the trial we used continuous audio sampling and video recording of the birds' behavior and our dictation to document the focal female's distance to the playback speaker (0-2, 2-4, 4-8, 8-16, and greater than 16 meters) and all female vocalizations. We later calculated the latency to respond, the duration of time a female responded, the duration of time each female spent vocalizing, a female's average and closest approach to the speaker, and how long the female was at or on the nest. At the end of the trial, we checked the nest to confirm nesting stage or, if the female had not responded, to determine if she remained on the nest through the trial. If the female was on the nest the trial was included and the distance to speaker was marked as a 24 m for the entire period she was on the nest to indicate a low aggressive response. If we could not confirm that the female was in the immediate area during the trail, the trial was not included.

We categorized female response to the simulated female territorial intrusion as (0) no response and/or left the area after being seen, (1) stayed on the nest for the entire trial or (2) responded aggressively at some point during the trial. Females that left the nest to respond and females that were already off and responded were grouped together in the "approached and responded" category. Additionally, to quantifying the strength of aggressive response, we used Principal Component Analysis (PCA) of the average distance, closest approach, duration of response, duration of vocal response, and latency to respond into a single "aggression score" (PC 1 which explained 74.41% of the variation in behavior, see Supplemental Materials Table 1) for each female. We interpret females' behavioral responses to these simulated territorial intrusions as aggressive signaling, not exploration or curiosity, based on previous descriptions of territorial aggression in female song sparrows (Arcese et al. 1988; Elekonich 1997). Additionally, though attacks and high intensity territorial aggression are rare in female song sparrows, we have observed several attacks in our study population in the past.

Maternal Care

To investigate the potential trade-off between female aggressive signaling and maternal care we monitored female nest visitation using radio frequency identification (RFID). After assessing female aggression, but before nestlings hatched, we placed a RFID system (Adelmen et al. 2014; Bridge and Bonter 2011) at the nest. The system consisted of an antenna wrapped in camouflaged colored electrical tape (for waterproofing) that we carefully placed around the external edge of the nest and attached to a battery hidden beneath the nest. The PIT tag is powered when it disrupts the magnetic field created by the antenna and the female's unique identification number is recorded by the microprocessor. Once the data is collected, we calculated daily nest visitation rates by dividing the number of visits a female made to the nest on a given day by the total time she provided care that day (time of last visit - time of first visits). Because song sparrows nest asynchronously, we necessarily collected data from different females on different days, over different durations depending upon when a nest was found, and during different stages of brooding and nestling care, which we accounted for in all statistical models (see below). This RFID system allows fine-scale monitoring (24 hours a day while the system is in place) of parental visitation during nestling provisioning. In total, we measured maternal visitation during nestling provisioning in 15 urban and 8 rural females from which we also had measures of aggressive signaling each for an average of 5 days/female. Nestling provisioning lasts for 10 days on average, and day 10 was the cut off for this study and most nestlings had fledged at that point.

Statistical Analysis

We conducted all statistical analyses using R (v. 3.6.1: R Core Team 2021). We used a chi-squared test of significance to examine the categorical responses of urban and rural females to conspecific female territorial intrusion. This allowed us to determine whether urban females were more likely to leave the area, stay on the nest, or respond aggressively in response to the simulated territorial intrusions compared to rural females. Additionally, to examine how female song sparrow aggressive signaling differs across rural and urban habitats, we used a linear model with female aggression scores (PC1) as the response variable, habitat type as the predictor variable, and year sampled, day of year, and nest stage (nest building, incubation, or brooding) as fixed-effects covariates.

We looked at the relationship between maternal visitation rates and maternal aggressive signaling with a linear mixed effects model fitted using the package "lme4" (Bates et al. 2015). We included maternal visitation rates as the response variable and aggression score as the predictor. We included habitat type, year sampled, nestling age, total number of nestlings, and day of year as fixed effects covariates and nest identification number was included as a random effect to account for repeated measures. For each linear model and linear mixed model, we examined the residuals to confirm normality. In the linear mixed effects models, we tested the significance of fixed effects using the lmerTest package (Kuznetsova et al. 2017), which estimates degrees of freedom with the Satterthwaite approximation.

Results

Urban and rural female song sparrows were equally likely to approach and vocalize in response to a simulated territorial intrusion by a conspecific female ($\chi^2_{1,95} = 1.46$, $p = 0.23$). Additionally, there was no difference in the behavioral strategy (i.e. whether to approach the simulated intruder, maintain parental behaviors, or flee the area) females used to respond to the simulated female conspecific intrusion ($\chi^2_{1,95} = 1.55$, $p = 0.46$). Specifically, 67% of urban females approached and vocalized compared to 54% of rural, while 12% of urban females and 18% of rural females remained on the nest. Only 22% urban females left the territory compared to 27% of rural females leaving.

However, the urban female song sparrows that did respond to simulated intrusions by conspecific females by approaching and vocalizing had significantly higher aggression scores than rural birds ($\beta = 0.89 \pm 0.43$, $t_{83} = 2.07$, $p = 0.04$; Figure 1). Additionally, female aggressive signaling during nest building was significantly higher than during incubation ($\beta = -2.39 \pm 1.01$, $t_{83} = -2.35$, $p = 0.02$), and generally decreased as the breeding substages progressed.

We found that female aggression scores were not significantly related to nest visitation rates regardless of habitat type ($\beta = -0.40 \pm 0.24$, $t_{1033} = -1.66$, $p = 0.11$; Figure 2), and there was no effect of habitat type on nest visitation rates during nestling provisioning ($\beta = 0.37 \pm 0.99$, $t_{1033} = 0.38$, $p = 0.71$). Additionally, there was a negative effect of nestling age on maternal visitation rates across habitat types ($\beta = -0.24 \pm 0.08$, $t_{1033} = -3.12$, $p = 0.002$).

Discussion

We found that urban and rural females were just as likely to respond to simulated female intrusions with aggressive signaling and approach. However, urban females expressed increased aggressive signaling during conspecific encounters compared to rural (Figure 1). This suggests that female behavioral strategies (i.e., whether females stayed on the nest, fled, or responded) do not differ between habitats, but that female aggression differs in magnitude of response as a function of habitat type. Several studies have demonstrated increased aggression in urban male songbirds, suggesting that urban habitats have features of resource distribution that make aggression beneficial. Very few studies have examined the relationship between urbanization and female aggressive signaling in any wildlife and the studies that have attempted to compare female behavior delivered mixed results. In songbirds, Miranda 2014 found no difference in aggression between urban and rural female European blackbirds (*Turdus merula*), though, when the sexes were pooled, rural birds expressed increased aggression, not urban. In contrast, Scheun et al. 2015 found that urban female African lesser bushbabies (*Galago moholi)* spent more time engaging in aggressive encounters than rural females, and that these encounters were likely food motivated. Finally, Kralj-Fišer et al. 2017 failed to find an effect of urbanization on female conspecific aggression in 3 species of orb-web spider (*Araneidae, Araneae*). The few species in which female behavioral responses to urbanization have been studied have such diverse life histories that we cannot draw conclusions about the impact of this form of environmental change on female behavior. Rather, these few studies highlight the gaps in our knowledge and the importance of research investigating the relationship between urbanization and female behavior.

In the present study we found that female aggressive signaling was higher in urban birds compared to rural, and there are several possible functional causes for this pattern. Urban sparrows have been shown to have larger territory sizes with decreased conspecific density compared to rural birds (Juárez et al. 2020), something also observed at our sites. Additionally, urban areas in this study are more fragmented, with anthropogenetic structures dividing the landscape, possibly making resources more easily defendable. Indeed, previous research has found that male aggression is directly related to food availability, and supplementation increased territorial aggression in males (Foltz et al. 2015). Additionally, female aggression has been shown to directly correlated with nest site availability in cavity nesting tree swallows (*Tachycineta bicolor*) (Rosvall 2008), but open cup species have been understudied in this regard. Finally, female aggression can function to assure social partner fidelity in the form of mate guarding (Dunn and Hannon 1991; Slagsvold 1993), something that could be selected for in a fragmented habitat with decrease conspecific interactions. Future studies could further investigate the relationship between habitat structure and female songbird aggressive signaling by manipulating food or nest site availability in open cup nesting songbirds across urban and rural habitats.

Though increased territorial aggression can be traded-off against parental care, we did not established a significant relationship between maternal care and a female's level of aggressive signaling (Figure 2). In a separate study of male song sparrows, we also did not find a significant relationship between paternal care and aggression (Unpublished data). This suggests that song sparrows are not under sufficient energetic pressure to drive a behavioral trade-off during the breeding season. The sample size in this aspect of our study is relatively low, as capturing and PIT tagging females during nesting is difficult. Future studies could manipulate parental effort, either through brood size manipulation or parental handicapping, across urban and rural habitats to directly test for a trade-off between parental care and territorial aggression in females. Studies of the costs of reproduction in urban habitats for individuals that may be more susceptible to the energetic demands of the breeding season, such as females, are important for fully understanding the costs of urbanization for wildlife.

Conclusion

The study of life history trade-offs are often heavily skewed towards males, and ecology in general and urban ecology in particular should consider life history trade-offs in females. Collectively, our results suggest that increased conspecific aggression is favored in urban habitats for female song sparrows as well as males, and that there is no detectable cost of this increased aggressive signaling to maternal care in urban habitats. This study adds to a growing body of literature documenting behavioral differences between urban and rural animals and highlights the importance of studying individuals of all age and sex classes in the study of urban ecology.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Acknowledgments

We thank Radford University, Virginia Tech's Kentland Farms and StREAM lab at Virginia Tech for allowing us to perform field research on their premises. We thank WA. Hopkins, J. McGlothlin, and IT. Moore for assistance on project development and comments on manuscript drafts. SJ. Lane received funding from the Virginia Tech Ralph E. Carlson Memorial Scholarship in Ornithology, the Global Change Center at Virginia Tech, the Graduate Student Assembly at Virginia Tech, the Virginia Society for Ornithology, the American Ornithological Society, and the Society for Integrative and Comparative Biology. This research was further supported by funding from National Science Foundation grant IOS 1652496 and IOS 2114288.

Figures

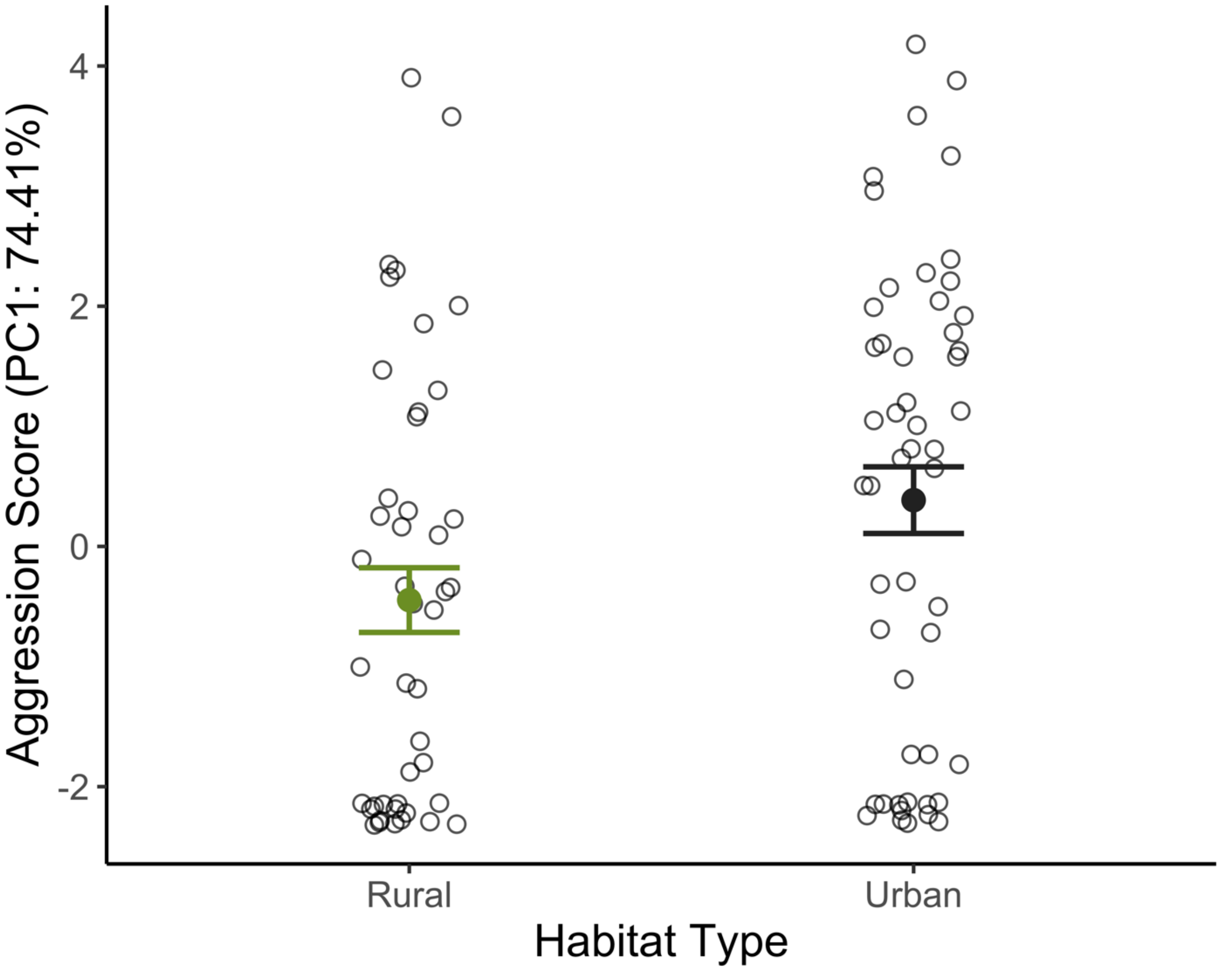

Figure 1 Female aggression scores (PC1) between urban and rural (green) female song sparrows. Urban females (51) had higher average aggression scores (0.39 ± 0.28) compared to rural females (44) who had and average aggression score of (-0.45 ± 0.27).

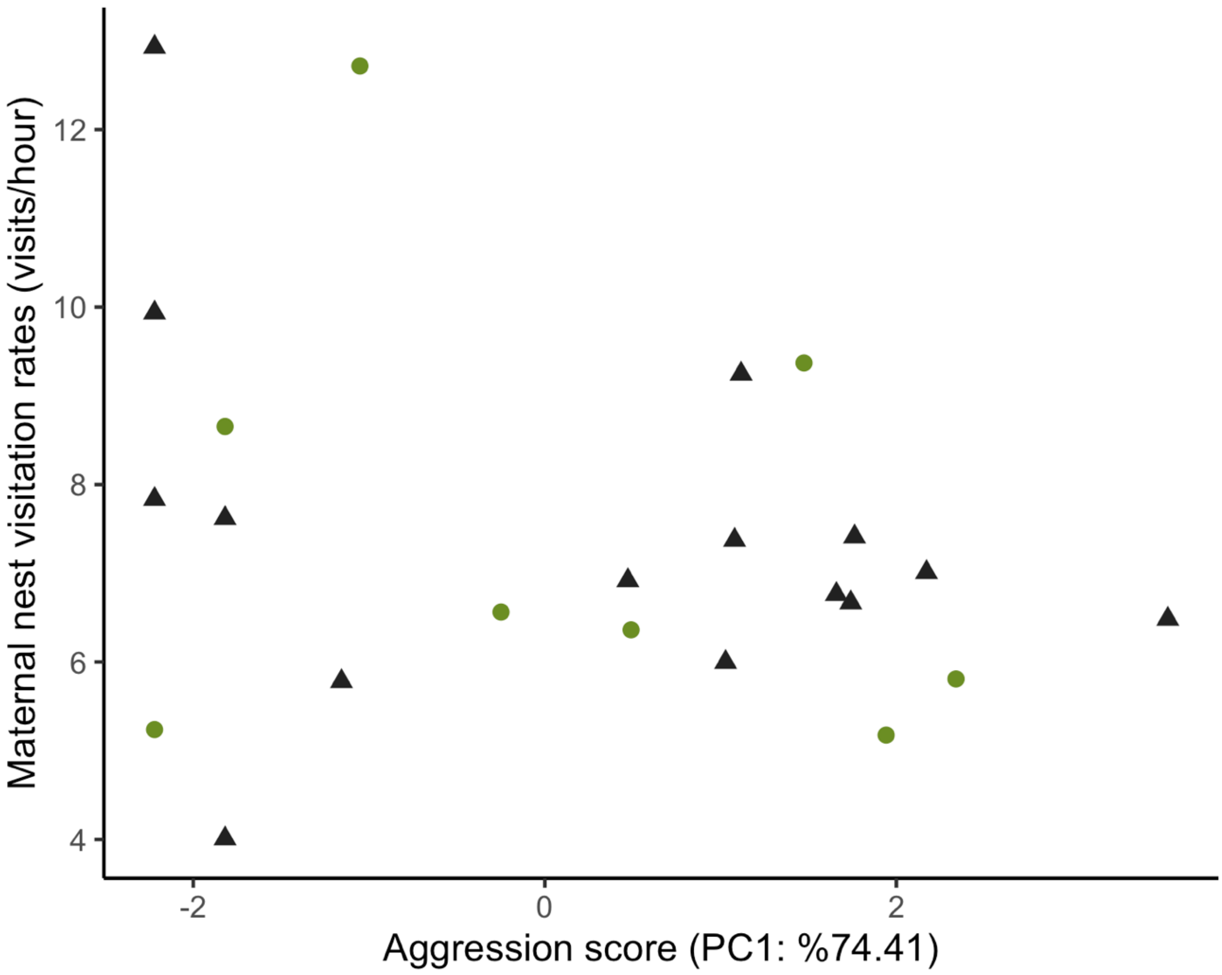

Figure 2 The relationship between female aggression (PC1) and maternal visitation rates of urban and rural female song sparrows. Across habitat types, there was a non-significant trend towards females with higher aggressive signaling having decrease nest visitation rates during nestling provisioning. Urban (triangles) females visited the nest 7.36 ± 0.25 times/hour compared to rural (green circle), who visited the nest, 7.25 ± 0.48 times/hour.

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Appendix B: Chapter 2 Supplementary Materials

Table B1: Results from PCA of female aggressive signaling behaviors

	PC1	PC2	PC3	PC4	PC5
Standard Deviation	1.9289	0.8374	0.55375	0.38829	0.34757
Proportion of Variance	0.7441	0.1403	0.06133	0.03015	0.02416
Cumulative Proportion	0.7441	0.8844	0.94568	0.97584	1

Chapter 3: Effects of urbanization and male aggression on parental care and life history outcomes in a wild songbird

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1 Abstract

Urban animals commonly express altered behavioral phenotypes compared to their rural counterparts. One well documented behavioral difference exhibited by many urban taxa, especially songbirds, is increased territorial aggression. However, we do not understand the consequences of this exaggerated urban male aggression on the fitness and behavior of males and their social partners. We explore the effects of increased urban male aggression on the life history traits, parental care, and offspring development of song sparrows (*Melospiza melodia*). We predicted that urban males would reduce paternal investment to increase territorial aggression and that these behavioral differences would force their social partner to increase investment to care for nestlings compared to rural sparrows. In contrast to our prediction, we found that aggressive urban males did not decrease care but visited the nest more often compared to rural males. Additionally, urban birds had higher reproductive metrics, a trend explained by higher rural nest predation rates. However, urban birds also had increased rates of brood parasitism by brown-headed cowbirds (*Molothrus ater*), which may negatively affect offspring survival after fledging. Our study adds to a growing body of evidence that urban habitats provide significant benefits to some generalist species and highlights the need to examine the effects of urbanization on the life history of diverse species.

2 Introduction

Life-history theory is structured around the idea that the evolution of suites of traits meant to maximize an individual's fitness work against each other to generate trade-offs (Reznick et al. 2000; Roff and Fairbairn 2007; Stearns 1992). With time and energy being limited, behavioral trade-offs occur when animals must strike a balance among traits that are energetically costly. The habitat in which an individual lives has been shown to modulate these reproductive behaviors, because the environmental context determines an animal's time and energy budget, and the extent to which behaviors constrain each other (Gunnarsson et al. 2005; Holtmann et al. 2017). Resource availability, conspecific density, predator presence, and heterospecific interactions are among the contextual factors that determine an individual's capacity to maximize fitness linked traits (Linden and Møller 1989; Martin 1995), and these factors are being altered by global anthropogenic change (Grimm et al. 2008b; McKinney 2008). Inhabiting urban habitats is imperative to the survival of many species, and to maintaining urban biodiversity as human expansion continues (Grimm et al. 2008b; McKinney 2002). However, urban habitats present wildlife with an altered abiotic and biotic landscape in which to live and reproduce (Chace and Walsh 2006; Grimm et al. 2008a) and may modify the trade-offs among life-history traits.

Urban habitats contain anthropogenic predators (e.g., domestic cats) (Loss et al. 2013; Rosenberg et al. 2019) that influence survival rates of both offspring and adults (Baker et al. 2008; van Heezik et al. 2010). These habitats are more fragmented than native habitats with different resource distribution and availability (Chace and Walsh 2006; McKinney 2006; Saunders et. al 1991) and obligate brood parasites tend to follow fragmentation and human disturbance (Brittingham and Temple 1983; Chace 2003; Faaborg et al. 2010; Robinson et al.

1995; Rodewald 2009) and may be more prevalent in urban communities. Despite the presence of these potential stressors, some species, termed 'urban adapters' (a species in which individuals from the same population compete for and maintain breeding territories in both urban and rural habitats; Blair, 1996; Bonier, 2012; McKinney, 2002; Shochat et al., 2006), have readily acclimated to urban environments. Within species comparisons of urban adapters examining individuals living in urban and rural habitats can provide insight into the acclimations that animals are making to novel urban habitats (Blair, 1996; Bonier, 2012; McKinney, 2002; Shochat et al., 2006).

Many species of songbirds have readily acclimated to cities, but breeding adults likely face new or exaggerated challenges not present in their native habitats. During the breeding season, male songbirds choose and defend territories with quality nesting substrates and resources needed to survive and rear young (Garcia and Arroyo 2002; Stamps and Krishnan 1997). In theory, the highest quality males should be the most aggressive and best able to defend the highest quality breeding territories (Garcia and Arroyo 2002; Otter and Ratcliffe 1996; Scales et al. 2013) leading to increased fitness (Otter and Ratcliffe 1996). Urban habitats often have fewer available breeding territories and resources compared to rural habitats (Juárez et al. 2020), and acclimation to urban habitats likely favors a more aggressive phenotype (Duckworth 2008; Foltz et al. 2015; Juárez et al. 2020). In fact, one behavioral pattern found in many urban dwelling taxa (Dammhahn et al. 2020; Lowry et al. 2013; Renthlei et al. 2017) but especially in songbirds, is increased male conspecific aggression (Atwell et al. 2014; Davies and Sewall 2016; Evans et al. 2010; Fokidis et al. 2011; Scales et al. 2011). Historically it has been thought that more aggressive males express decreased social partner investment and reduce paternal care (Clutton-Brock et al. 1982; Duckworth 2006; Stearns 1992; Stoehr and Hill 2000). While an exaggerated aggressive phenotype may facilitate territory acquisition and/or tenure in urban habitats, investment in aggressive behavior could result in a trade-off with other energetically costly behaviors, such as parental care (Duckworth 2006; Ketterson and Nolan 1999; Ketterson and Nolan 1994; McGlothlin et al. 2007). For socially monogamous urban songbirds, any trade-offs in breeding behaviors (i.e., territoriality and parental care) can have consequences for social partners because mates cooperate in rearing young (Gunnarsson et al. 2005; Holtmann et al. 2017). How social pairs resolve these trade-offs can have significant consequences for adults and the development and survival of their offspring (Duckworth 2006; Grimm et al. 2008; Marzluff 2001; Scolozzi and Geneletti 2012).

Shifts in paternal behavior can have consequences for social mates in species that provide biparental care. Many territorial songbird species establish social partnerships during the breeding season to provide biparental care to their offspring (Arcese 1989; Griffith et al. 2002; Westneat 1987). Social monogamy allows partners to split the energetic demands of reproduction, so the burden of care does not fall on one individual (Griffith et al. 2002; Møller and Birkhead 1993). In north temperate songbirds, the predominant energetic assistance provided by males is offspring provisioning and aggressive behaviors, such as territoriality and nest defense (Duckworth 2006; Ketterson and Nolan 2013; Saino and Møller 1995). Between socially monogamous but genetically polygamous social pairs, only females have guaranteed paternity, and often show increased reproductive investment compared to males (Arcese 1989; Griffith et al. 2002; Westneat 1987). The energetic demands of increased aggression in urban males may constrain their ability to provide paternal care, affecting offspring fitness and the energetic requirement of their social partner in the form of increased maternal care. Therefore, if more aggressive urban males are sacrificing paternal behaviors increase territoriality, it may require increased maternal care to maintain offspring development and fledging success.

Here, we investigate the effects of urbanization and associated increase in male aggression on the parental care provided by males, the care provided by their mates, and the development and fledging success of their offspring by comparing adult behavior and nestling outcomes among wild song sparrows (*Melospiza melodia*) in urban and rural habitats. Song sparrows are a commonly studied urban adapter that, like many songbird species, are open cup nesting altricial species, where biparental parental care is especially vital (Martin and Briskie 2009; Nice 1943; Roff et al. 2005; Smith and Roff 1980). We hypothesized that urbanization alters behavior of males such that they resolve trade-offs differently than their rural counterparts. We then examined the consequences of these behavioral differences on their social partners and offspring. Specifically, if more aggressive urban males provide less paternal care than their rural counterparts, then their social partners are expected to compensate to maintain offspring fitness or, if they cannot, nestling growth and survival should be reduced. We predicted that urban males would provide less care compared to rural males. We also predicted that maternal care would be positively correlated with social partner aggression, and negatively correlated with paternal care. Finally, we predicted urban nesting success would be lower, and urban offspring would be underdeveloped compared to rural birds.

3. Methods

Parental behavior

Subjects

To examine the relationship between male aggression and parental care, in the breeding seasons of 2018 -2021 we measured male aggression in 42 urban males and 16 rural males from 6 previously established sites in Southwestern Virginia, USA that vary along an urban-rural gradient (Davies et al. 2016) following protocols established by Davies et al. (2018). To evaluate the levels of urbanization, Davies et al. 2018 used an automated system to assign an urbanization score to each site using the protocols described in Seress et al 2014 (for full details see Davies et al. 2018). After we located a nest and established the breeding stage, we measured male territorial aggression toward a conspecific song. We performed behavioral trials between April and July of each breeding season (see supplemental materials for a detailed figure outlining our methods timeline).

To monitor social partner parental care, we captured adult birds with mist nets between 0500- and 1115-hours from March through July of each year. We captured males by targeting them immediately after simulated territorial intrusions, while females were captured when leaving or returning to the nest. To avoid nest abandonment from parental capture, we allowed all active nests to reach at least mid- to late-incubation before capture, did not attempt to catch a social pair (a) for more than 120 min/day (b) during inclement weather or (c) on sequential days. We took morphological measurements from the adults (body mass, tarsus, wing chord and tail lengths, bill width, depth and length, brood patch and cloacal status and fat deposits) and banded them with a unique color band. Before releasing each bird, we fitted a passive integrated transponder (PIT) tag (2.12x10mm; CYNTAG, Inc; Item#: 601205-2248) to the tarsometatarsus of each adult (including social partner when both were captured) following the methods established in Bridge and Bonter 2011 to monitor parental care. The PIT tags are small coils of magnetic wire programed with a unique 10-digit code that can be read by any radio frequency identification (RFID) system (Bridge and Bonter 2011; Farr et al. 2021). We gave every bird a unique combination of color bands and multicolored heat shrink tubing (Figure 1a) to allow us to visually identify individuals using binoculars.

Male Aggression

During trials we located focal males based on location of spontaneous singing relative to the active nest and/or in response to brief (< 1 min) playback. Once located, we exposed a male to 6 min of playback with a 3D printed song sparrow mount placed at the approximate center of the territory (Beck et al. 2019). Each playback consisted of 2 song types recorded from the same male designed to approximate a male's natural song (Davies et al 2016). To assess male territorial aggression during each playback, we recorded distance to speaker/mount, number of broadcast songs, number of low amplitude songs (soft songs), and number of wing waves (Akçay et al. 2011; Davies and Sewall 2016; Davies et al. 2018). To calculate the average distance to the speaker during the trials, we recorded bin distance from the speaker (0-2, 2-4, 4-8,8-16, and >24m) every 5 seconds for the entire 6 min behavioral trail, then calculated the average distance from the speaker (Hyman et al. 2004; Davies et al. 2018). We combined all the territorial response data into a single 'territorial aggression score' using Principal Component Analysis (PCA; See supplementary materials for table of loadings). Male aggression has been shown to be repeatable within individuals during a breeding season (Davies and Sewall 2016), though territorial aggression declines over the season. Therefore, we used a single trail to quantify aggression but include day of year in all analysis.

RFID and parental care

To monitor parental feeding rates in 2018 – 2021, we placed a RFID system at the nest (For details on function, form, and construction of RFID systems for monitoring wild bird populations see Adelman et al. 2014; Bridge and Bonter 2011) on the days leading up to nestling hatch. The system consisted of an antenna wrapped in camouflage electrical tape (for waterproofing) that was carefully placed around the external diameter of the nest (Figure 1b). The antenna was attached to a battery-powered microprocessor (E. Bridge; GEN 2 RFID Reader and Data Logger, 2012; Bridge and Bonter 2011) with a two GB memory card (hereafter we refer to the microprocessor and memory card as the "board"). The board and battery were housed in a waterproof container that was hidden near the nest. When the PIT tag disrupts the magnetic field created by the antenna, it is powered on and transmits a unique identification number which is recorded by the board as long as the PIT tag is within its range (here the range is the internal area of the nest cup plus a few centimeters outside, above, and below the bottom of the nest cup). Batteries for the RFID system were changed every 2 – 3 days.

This RFID system was set with a read interval of 6 seconds and allows for continuous fine-scale monitoring of parental care during the entire nestling period. All RFID data were inspected visually for quality and accuracy. We used videos to spot-check RFID data by visually recording behavior and matching it to data recorded by the boards. We considered a lag time of >1min between reads of a PIT tag number as a male leaving the nest. This was marked as an 'OFF' time in the data. To calculate the number of nest visits made by an individual, we summed the number of OFF visits recorded in a day. Females move frequently when on the nest during the early nestling stage, so we increase the OFF time to >2 min to be more conservative in analyzing female visitations. To calculate "total time" an individual provided care for a given day, we subtracted the first and last read for that individual for the day. We used the number of visits and the total time on the “total time” for each day to calculate visitation rate (visits / (time of final visits in the day – time of first visit of the day) for each parent each day of the nestling period. Additionally, we calculated the duration of each visit (time/visits) and nest activity/day following similar methods. In total, we measure parental care in 73 males (56 urban and 17 rural) and 40 females (24 urban and 16 rural) from 72 urban nests and 24 rural nests. Parental care was monitored for 24 hours a day throughout the breeding attempt. Not all nests survived the breeding attempts, and some nests were located, or parents were captured after hatch.

Monitoring nesting success and offspring development

Nest searching and monitoring

To examine the effects of urbanization on the fitness of wild song sparrows, we located and monitored nests of song sparrows in urban and rural habitats throughout 5 breeding seasons. In the spring and summers of 2017 to 2021 we searched for and monitored song sparrow nests at the previously mentioned field sites. We searched habitat types evenly (2 days searching urban, 2 days searching rural) to control for nest searching effort. Nest searching was conducted primarily in the early morning (0500 to 1100) during peak parental behavior, from early May until late July in 2017, and from early March to late July in 2018- 2021. For analysis we only included one nest from each pair that was closest to the mean lay date for the year. In total, we found and monitored 166 nests in urban habitats and 119 nests in rural habitats.

We located nests primarily through behavioral observations of adults and systematic searching of known territories and nesting substrates (Martin and Geupel 1993). When we located nests during incubation, a minimum of two eggs were candled to establish incubation stage and target hatching date. After we located a nest, we limited the amount of time spent at the nests and number of visits to the nests to reduce nest abandonment. When possible, we established nesting stage through behavioral observation of parents (e.g., parents bringing food during nestling stage, female with nest material during build, or female on the nest during incubation). Additionally, we only visited nests to establish vital developmental stages (lay date, onset of incubation, and hatch date), monitor offspring development, and conduct adult behavior assays and capture. We used previously published life-history data for this species to estimate dates for events (i.e., nest building, lay and incubation duration) that occurred before a nest was found (Smith and Roff 1980; Sogge and Van Riper 1988).

Nest success was defined as at least one song sparrow nestling fledging from the nest. Nest failure was defined as complete nest predation (all eggs gone from the nest, all nestlings gone while still altricial), parental abandonment, or an anthropogenic form of nest destruction. If nest success or failure was not recorded on the RFID, the date of nest completion was estimated as the midpoint between the last and final nest visits (Mayfield 1961). To control for stage location bias (nests found in late stages overestimate nesting success), daily nest survival rates and nest survival rates for each habitat were calculated Mayfield estimations following previously established methods (Mayfield 1961). We visually inspected all final clutches for brood parasitism by brown-headed cowbirds (*Molothrus ater*), using previously established methods to distinguish host eggs from brown-headed cowbirds (Smith and Arcese 1994). If a nest was found after hatch, we use visual identification and morphometrics to identify brood parasitism (Pyle 1997).

Offspring development

To examine the effect of urbanization on offspring development we monitored nestling development across habitat types. Across all years we measured 349 urban nestlings and 128 rural nestlings from 118 urban nests and 47 rural nests. To monitor offspring development and establish growth curves we excluded cowbird nestlings and measured nestling sparrow mass, wing chord, beak, head, pin, tail, and tarsus on hatch day (day 0 -1; Pyle 1997), or when the nest was found. To ensure accuracy, each measurement was taken 5 times, the highest and lowest measurement were dropped, and an average was taken from the remaining 3. We remeasured morphometric variables every 2 – 3 days until fledge. If we located a nest after the clutch had hatched, measurements were taken on the day it was found and nestling age was estimated from morphometric variables (Jonsomjit et al. 2007; Pyle 1997).

Statistical analysis

Behavior

We conducted statistical analyses using R (R Core Team 2019, v. 3.6.1). We analyzed all parental care and male aggression data using linear mixed effects models (LMM). We fitted all LMM's and generalized linear mixed effects models (GLMM) using the package "lme4" (Bates et al. 2015) and we examined the residuals from each model for normality. We tested the significance of fixed effects from the LMMs using the lmerTest (Kuznetsova et al. 2017) package, which estimates degrees of freedom (*df*) with the Satterthwaite approximation. We analyzed the effect of habitat on paternal care, maternal care, and the correlation between paternal care and aggression in separate LMMs using a data set that included all adults monitored during this project. In each model, visits/hour (by either the male or female depending on the model) was used as the response variable. For the two models examining paternal and maternal care, habitat type was a categorical fixed-effect. For paternal care and aggression, we included male aggression score (PC1, which explained 53.19% of the variation in behavior, see Supplementary Table S1) and habitat type as the predictor variables with an interaction indicated. For maternal care and male aggression analysis, we included maternal visits/hour calculated for each day as the response variable with social partner's aggression score and habitat type as the predictor variable with an interaction indicated. We created a final LMM to examine the relationship between social partner parental care that only included the nest where we had parental care from both social partners (41 pairs). For this model, we averaged male and female social partners visitation rates for each day, then included female care as the predictor variable, with male care and habitat type as fixed-effects. For all behavioral analysis we included age of the nestlings (day 1 – 10), total number of nestlings in the nest (including brood parasites), Julian day, and year sampled as fixed-effect covariates. Paternal care did not vary with the presence of a brood parasite in the nest, and the covariate was dropped from all parental care models, but brood parasite nestlings were included in all brood sizes. We included the unique nest ID as the random effect in each model to control for autocorrelation within the nest and for repeated measure of continuous recording of parental care.

Fitness measures and nest success

To analyze patterns of nest success, nest predation, and brood parasitism by brown-headed cowbirds between urban and rural habits we used separate linear model (LM)'s fitted to a binomial distribution. For each model we included nest success or failure, presence or absence of nest predation, and presence or absence of brood parasitism respectively, as the binomial response variables. We used habitat type, year, and lay date as fixed-effect covariates in the models. We further analyzed fitness measures of fledging success and hatching success between urban and rural habitat using generalized linear models (GLMs) fitted to a negative binomial distribution. In each model, fledging success or hatching success was the response variable, with habitat type, lay initiation date and year included as fixed-effect covariates. Finally, we examined a commonly used fitness metric, lay date, between habitat types using a LM (Winkler et al. 2020). We included Julian lay date as the response variable and habitat type, brood parasitism, and year as fixed-effect covariates in the model.

Nestling Growth

To examine the difference in nestling growth between habitat types we used two separate LMMs with morphological measures (either nestling tarsus length or mass) as the response variable in each model. We included habitat type and nestling age as fixed-effects with an interaction indicated. Julian date, number of nestlings in the nest, brood parasitism, and year were also included as fixed-effect covariates. To control for autocorrelation between nestlings in the same nest, and repeated measures of measuring nestlings on multiple days we included the unique nest ID as the random effect.

4. Results

Male aggression and parental care between urban and rural habitats

Male nest visitation rates significantly increased with the total number of nestlings present in the nest ($\beta = 0.45 \pm 0.11$, $t_{495.43} = 4.05$, p = <0.0001) and nestling age ($\beta = 0.19 \pm 0.03$, $t_{545.23} = 6.411$, p = <0.0001) across habitat types. However, independent of brood size and nestling age, urban males visited the nest significantly more often compared to rural males ($\beta =$

0.92 ± 0.03, $t_{79.15} = 2.20$, p = 0.04; Figure 2; Random effects: among-nest variance = 2.35, residuals variance = 2.45). Contrary to our predictions, we found no relationship between male aggression and nest visitation rate through the brooding period (β = 0.09 ± .62, $t_{54.35} = 0.15$, p = 0.89; Figure 2) nor did we find a relationship between male aggression and maternal visitation rates (β = -0.81 ± 0.67, $t_{29.12} = -1.20$, p = 0.24; Random effects: among-nest variance = 0.70, residuals variance = 2.04). Males in both habitat types never visited the nest outside of daylight hours. While urban males visited the nest more frequently than rural males, females did not. There was a trend for increased maternal visitation in urban habitats (β = 0.66 ± 0.33, $t_{48.39} = 1.99$, p = 0.05; Figure 4; Random effects: among-nest variance = 0.80, residuals variance = 1.94) and a significant negative relationship between female visitation rates and nestling age (β = -0.08 ± 0.03, $t_{315.08} = -2.49$, p = 0.01), but female visitation rates did not vary with the number of nestlings present in the nest (β = 0.08 ± 0.12, $t_{224.27} = 0.64$, p = 0.52). Finally, there was a significant positive relationship between daily social partner nest visitation rates across habitat types (β = 3.33 ± 0.05, $t_{231.20} = 7.17$, p =<0.0001; Random effects: among-nest variance = 0.56, residuals variance = 1.53; Figure 5) and there was no difference in this relationship between habitats.

Offspring development and success between urban and rural habitats

Overall, urban nestlings had significantly higher body mass (β = 0.80 ± 0.35, $t_{303.3} = 2.33$, p = 0.02; Figure 6; Random effects: among-nest variance = 1.76, residuals variance = 2.73) and tarsus length (β = 0.92 ± 0.36, $t_{293.4} = 2.51$, p = 0.013; Random effects: among-nest variance = 2.20, residuals variance =2.46) compared to rural across the nestling period. However, urban nestlings grew significantly slower than rural nestlings between hatching and fledging (Mass: (β

= -0.19 ± 0.05, t_{1033} = -4.11, p = <0.0001; Figure 6; Tarsus (β = -0.18 ± 0.05, $t_{1034.0}$ = -3.87, p = 0.0001)). Additionally, nestling body mass (β = -0.96 ± 0.29, $t_{160.2}$ = -3.24, p = 0.0014; Figure 7; Random effects: among-nest variance = 1.76, residuals variance = 2.73.) and tarsus length (β = -0.83 ± 0.32, $t_{157.2}$ = -2.61, p = 0.0099; Random effects: among-nest variance = 2.25, residuals variance = 2.49) were significantly lower in nests containing brown headed cowbird nestlings compared to those without.

The fitness effects of breeding habitat type and nest density

Nest success rates were significantly higher in urban habitats compared to rural (β = 2.03 ± 0.50, z_{152} = 2.63, p = <0.0001; Figure 8c) but success rates varied across years (β = -0.42 ± 0.16, z_{152} = -2.64, p = 0.008; Figure 8c). Similarly, hatching success (β = 0.95 ± 0.25, z_{140} = 3.84, p = 0.0001) and fledging success (β = 1.02 ± 0.34, z_{122} = 3.02, p = 0.003) were significantly higher in urban habitats compared to rural. Nest predation rates were significantly higher in rural habitats compared to urban (β = -2.37 ± 0.44, z_{154}= -5.35, p = <0.0001; Figure 8a) and nest predation rates also expressed inter-year variation (β = 0.34 ± .002, z_{154} = 2.14, p = 0.03; Figure 8a). Brood parasitism by brown headed cowbirds was significantly higher in urban habitats compared to rural (β = 3.25 ± 0.78, z_{154}= 4.18, p = <0.0001; Figure 8b). We found that lay date was significantly earlier in urban habitats compared to rural (β = -18.97 ± 3.58, t_{132} = -5.30, p = <0.0001), with rural birds starting to lay on May 27th ± 2.27 days (64 nests with confirmed building or lay dates across 5 years) and urban around May 11th ± 1.97 days (110 nests with confirmed building or lay dates across 5 years). When nest predation pressure was experimentally tested across habitat type with our artificial nest experiment designed to test for effects of nest density, we found that urban habitats had significantly lower nest predation rates compared to rural (β = -1.19 =, z = -3.14, p =0.0017). However, there was a nonsignificant trend towards increased nest predation in higher density transects (β = -0.53, z =-1.96, p =0.0501).

5. Discussion

Time is a limiting resource for seasonal breeding species that have a narrow window to mate and raise their offspring, and decisions on how individuals allocate time can be influenced by the environment. For example, greater male aggression seen in urban habitats may shift an existing trade-off between paternal care and territorial defense (Pryke and Griffith 2009). Decreased male care could increase maternal investment. However, if this requirement is unsustainable, offspring development and survival could decrease compared to individuals in rural habitats. Contrary, to this theoretical framework, we found no evidence that urban male song sparrows sacrificed parental visitation for territorial aggression. Urban males did not have lower visitation rates than rural males at any nestling age or time of day (Figure 2). In fact, we found that the more aggressive urban males visited the nest more often than their rural counterparts and began feeding earlier in the day (Figure 2). Additionally, there was no correlation between male aggression and parental visitation rates (male or female) within and across habitats (Figure 3). This refutes both the idea that urban males are facing a trade-off between territorial defense and parental care, and that urban habitats are detrimental. Previous research has shown that urban sparrows have decreased conspecific density and increased territory size compared to rural (Davies et al. 2016; Juárez et al. 2020). With larger territories to defend, but decreased conspecific density, urban males likely have differing time and energy budgets compared to rural, but neither rural nor urban seem to be under sufficient energetic strain to force a behavioral trade-off between our measures of aggression and paternal care.

Though female visitation rates overall were not significantly different across habitats, there was a nonsignificant trend for urban females to visit the nest at higher frequency (Figure 5). Specifically, urban females increase visitation rates during the day compared to rural, while rural females are more active at night compared to urban females (Figure 5). Our findings align with research on cavity nesting species showing urban females have increased incubation bout rates and decreased time on the nest (Heppner and Ouyang 2021). Future studies looking at daily patterns of behavior across urban and rural habitat in species with differing life-history traits could elucidate what behaviors coincide with this variation in diurnal and nocturnal nest activity between habitats in brooding females.

We also found a strong positive relationship between parental care provided by social partners (Figure 5). Females that visited the nest more often each day were paired with males that did the same. Collectively, these parental care findings also refute the hypothesis that urban females are compensating for their social partners, because males aren't compromising care for aggression, and seem to match their reproductive effort with that of their partner. This behavioral matching raises the hypothesis that song sparrow social pairs express assortative mating and/or matching of parental behaviors within the brooding periods (Westneat et al. 2011). This has been seen on other species, where male plumage is an indication of their paternal abilities (Wolfenbarger 1999). Future studies that manipulate male sexual signals and social pair structure could examine female choice and behavioral matching in these socially monogamous sparrows.

In support of our hypothesis that urban habitats would be detrimental to nestling, we found that urban nestling growth (measured as mass and tarsus growth) was slowed compared to rural (Figure 6). However, urban nestlings were significantly smaller at fledge, but on average, were larger across the nestling period compared to rural. We believe this counterintuitive result is due to higher rates of brood parasitism by brown-headed cowbirds in urban habitats (Figure 8b). Nestlings that hatch later often die of malnutrition in parasitized nests, and only the largest, most robust host nestlings survive to fledge, reducing the overall growth trajectory of host nestlings. In support of this, we found that urban nestling growth metrics were significantly lower in nests containing brown-headed cowbird nestlings (Figure 7), and differences in nestling mass and tarsus length between parasitized and unparasitized nests near fledged appear reduced (Figure 7). Obligate brood parasites, such as brown-headed cowbirds, lay their eggs in the nest of a host species, forcing the host to raise the nestling, often to the detriment of the parent and their offspring (Faaborg et al. 2010; Ortega et al. 2005). The significant effects of brood parasitism on nestling growth suggests that slowed urban nestling growth is not due to reduced paternal visitation, but rather an effect of the high rates of parasitism in urban habitats. We found that urban nests had higher daily and annual survival rates across all five years compared to rural (Table 1). Additionally, we found urban song sparrows had greater nestling (Figure 8c), hatching and fledging success, and began nesting significantly earlier compared to rural. This higher nestling survival was driven by significantly lower nest predation rates compared to rural (Figure 8a).

Our findings add to a growing body of evidence that despite increased predator presence in urban habitats, nest predation rates decrease in urban settings compared to rural habitats (Eötvös et al. 2018; Fischer et al. 2020; Gering and Blair 1999; Shochat et al. 2006). Predators can exploit anthropogenetic food sources such as trash cans and human food supplementation in place of spending time and energy searching for nests (Fischer et al. 2020; Seress and Liker 2015). Additionally, predators like snakes, whose conspecific density decreases in urban habitats, are known to increase in prevalence in areas undisturbed by urbanization (Klug and

Jackrel 2010). The difference seen in nest predation pressure between habitat types could also influence the differences in urban parental visitation rates (Eggers et al. 2006; Lyon and Montgomerie 1987; Martin and Ghalambor 1999), as previous research has experimentally shown that increased nest predation pressure decreased nest visitation rates (Mouton et al. 2020; Ghalambor and Martin 2002). These previous studies indicate urban birds can visit the nests more often as they are less likely to attract predators, providing further evidence of the benefits of urban habitats to some urban generalists. Also, adults in rural habitats could augment the decreased visitation rates by bringing larger or higher quality food material. Collectively these results suggest that certain urban generalist have significantly greater nesting success in urban habitats compared to rural. Future studies that experimentally manipulate paternal effort, brood size and parasitism, and nest predation pressure would help disentangle the effects of altered brood size and decreased nest predation risk in urban habitats from urban male quality.

Conclusions

There is an underlying assumption in many urban ecology studies that urban habitats are detrimental to animal fitness compared to the rural, less disturbed environments. Our data join an increasing number of studies showing this is not the case for all species, and that urban habitats provide benefits to species that are able to invade these novel environments (Chamberlain et al. 2009; McKinney 2008; McKinney 2002). We found that more aggressive urban male song sparrows did not sacrifice parental care for territorial aggression. In fact, they visited the nest more often than their rural counterparts, a pattern also observed in females though the relationship wasn't significant. These behavioral differences could be the result of decreased nest predation pressure in urban habitats. Moreover urban males aren't compromising paternal care, measured as visitation rates, for their increased aggression, song sparrows either do not face such a trade-off or they are released from it in urban habitats. Consequently, their social partners aren't suffering increased energetic demands to care for young. We did observe a negative effect of urban habitats on the growth of urban nestlings, a result likely explained by the prevalence of brown-headed cowbirds in urban habitats compared to rural. However, the long-term effects of these increased rates of brood parasitism on the adult and nestling fitness remains to be explored. Finally, we observed positive nestling outcomes in urban birds compared to rural in the form of decreased nest predation rates and increased fledging success. Understanding the effects of urbanization requires understanding community dynamics, including the interactions between brood parasites, predators, and their hosts. Previous studies found that urban adapters, such as the songbirds in this study, receive benefits such as increased food, water, maintained breeding substrates, and respite from predators (Chamberlain et al. 2009; McKinney 2002; Reynolds et al. 2019; Shochat et al. 2010; Soulsbury and White 2016; Stracey and Robinson 2012). Collectively our findings align with these studies, and suggest that, if given the space and opportunity to breed and reproduce, some generalist urban adapters and the species that rely on them can flourish in urban environments.

Acknowledgments

We thank Radford University, Virginia Tech's Kentland Farms, and StREAM lab at Virginia Tech for allowing us to perform field research on their premises. We thank WA. Hopkins, J. McGlothlin, and IT. Moore for assistance on project development and comments on manuscript drafts and A. Gamero, and CE. Moore, for assistance in the field. SJ. Lane received funding from the Virginia Tech Ralph E. Carlson Memorial Scholarship in Ornithology, the Global Change

Center at Virginia Tech, the Graduate Student Assembly at Virginia Tech, the Virginia Society for Ornithology, the American Ornithological Society, and the Society for Integrative and Comparative Biology. VN. Brewer and CR. Linkous received independent funding from the Multicultural Academic Opportunities Program, the Global Change Center at Virginia Tech and from the Howard Hughes Medical Institute's 2014 Science Education grant 52008103 to New Mexico State University. This research was further supported by funding from National Science Foundation grant IOS 1652496.

Tables

	Rural (%)			Urban (%)		
Year	Daily Survival	Apparent Survival	Mayfield Estimate	Daily Survival	Apparent Survival	Mayfield Estimate
2017	92.75	33.33	17.73	96.55	63.16	44.61
2018	88.43	12.50	5.91	97.16	66.67	51.59
2019	90.50	12.50	10.06	97.03	56.90	49.95
2020	88.70	7.14	6.34	94.91	31.71	30.07
2021	82.16	13.16	1.09	96.32	37.21	42.18
Cumulative	88.81	14.50	**6.53**	96.55	50.79	**44.57**

Table 1: Daily, apparent, and Mayfield estimations (Mayfield 1961) of nest survival rates between urban and rural song sparrows from 2017 – 2021

Figures

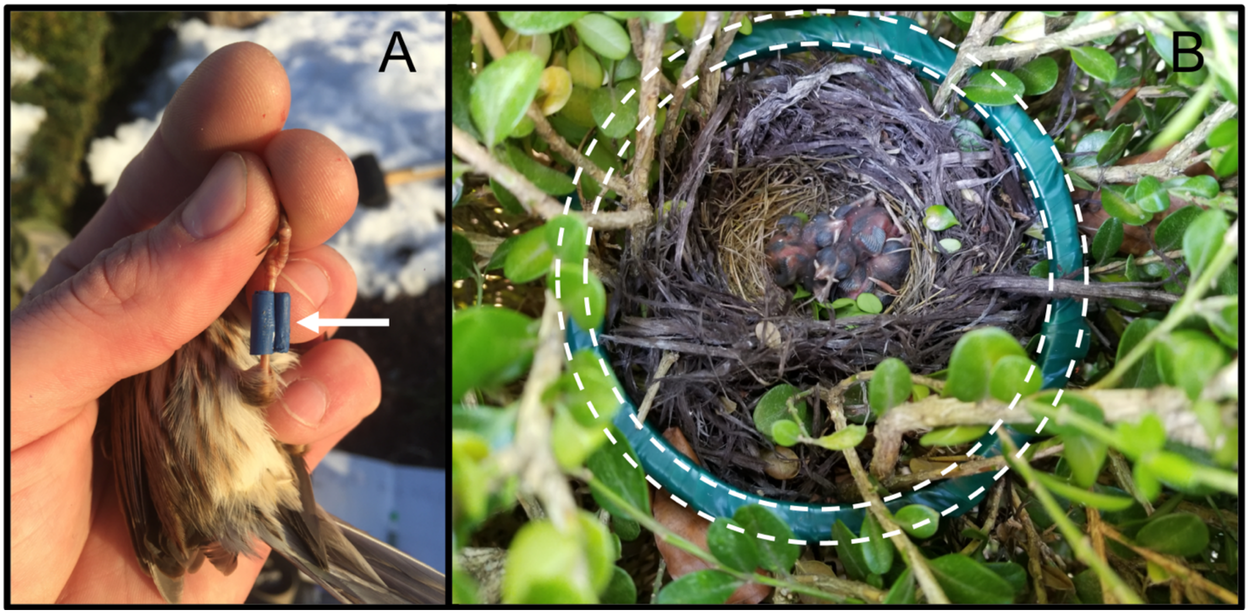

Figure 1: A PIT tag attached to the tarsometatarsus of a song sparrow (A) and an antenna fitted around a song sparrow nest (B) during the 2018 field season. When the magnetic coil built into the PIT tag disrupts the magnetic field created by the antenna, the duration of the visit is recorded, time stamped and stored by the microprocessor hidden nearby in a waterproof container.

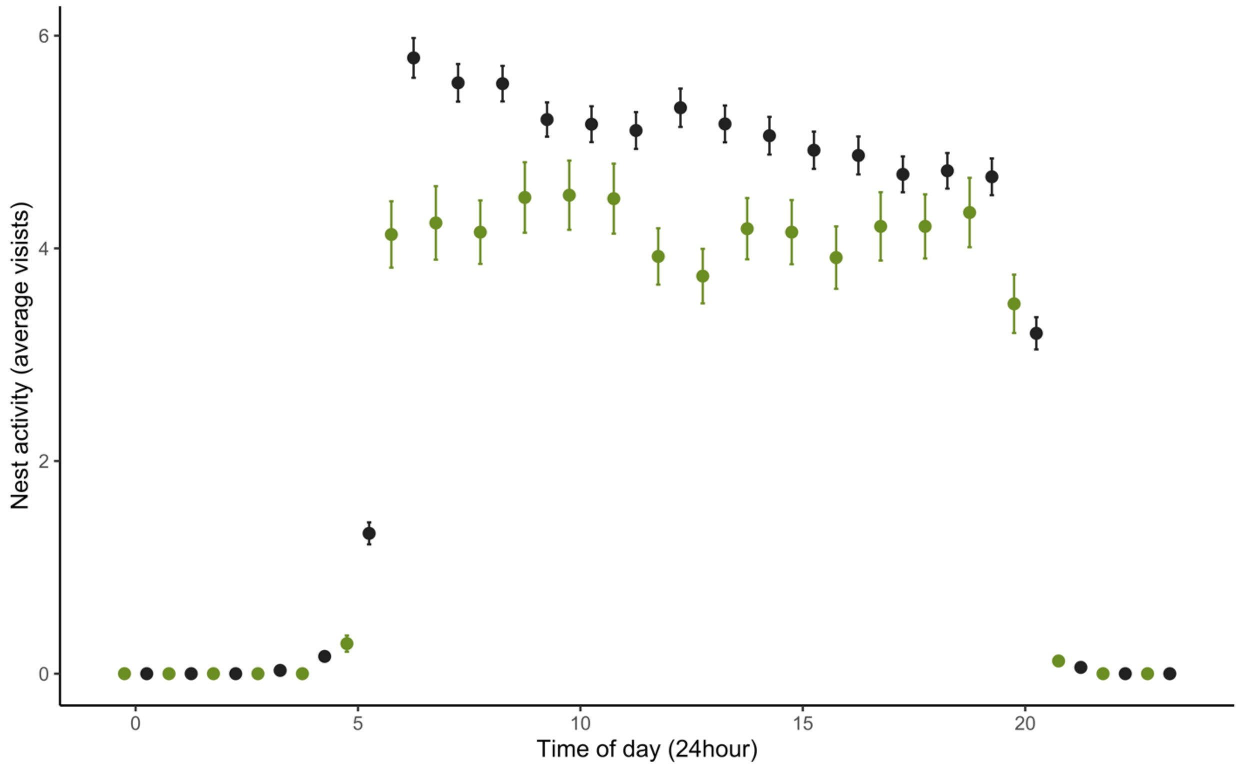

Figure 2: Paternal visitation between urban and rural (green) habitats during the nestling period (day 0 – 10) Urban males (56) visited the nest significantly more often (5.66 ± 0.11 times/hour) during nestling provisioning compared to rural males (17) that visited the nest on average 4.70 ± 0.18 times/hour. During these visits, urban males spent on average 7.60 ± 0.31 minutes per visits compared to rural, who spent on average 7.72 ± 1.04 minutes per visit. Each time point is averaged from 388 urban observation (days) and 92 rural observations (days).

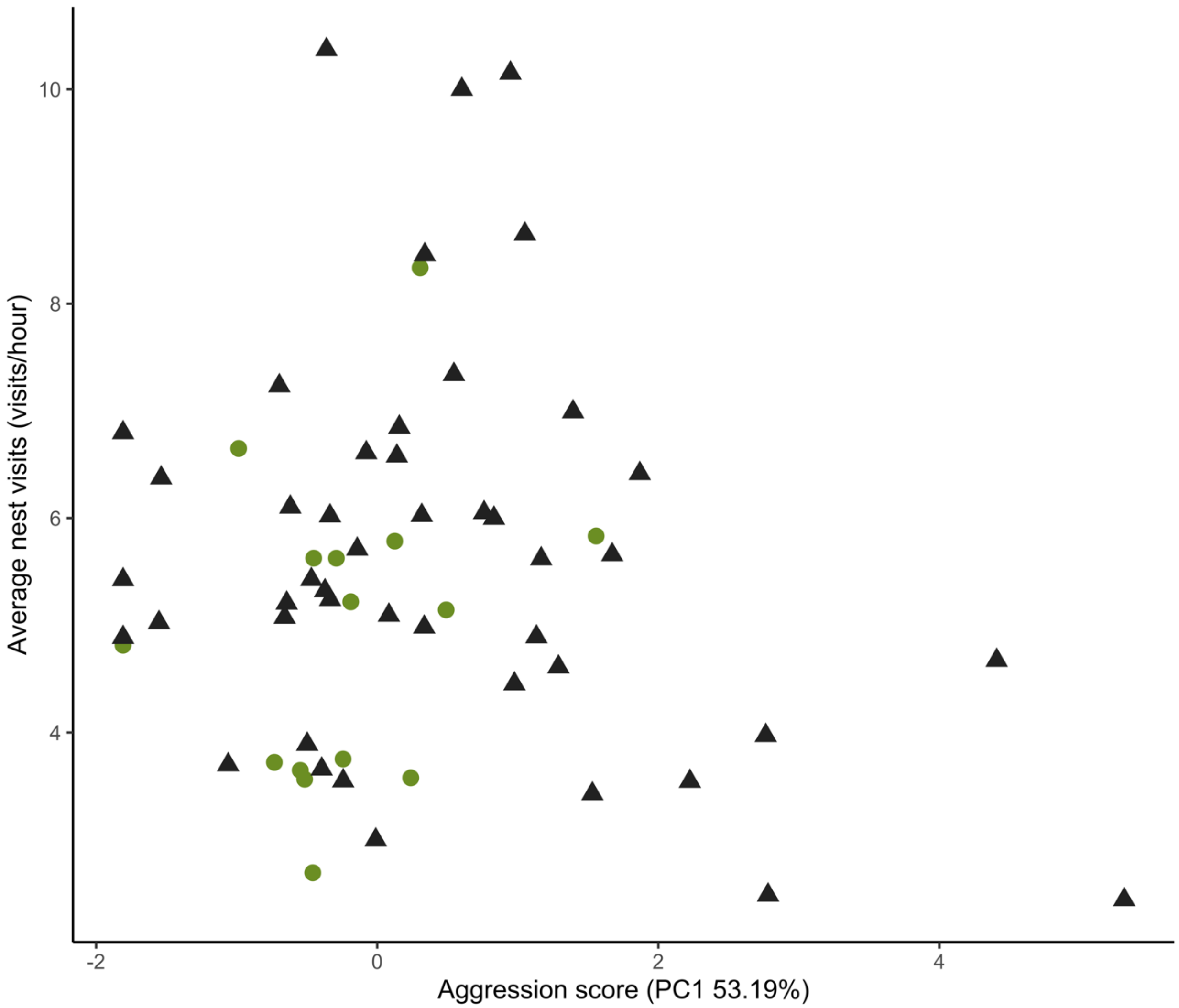

Figure 3: Male aggression in urban (black triangles) and rural (green circles) song sparrows was not correlated with paternal care. Urban males (46) had an average aggression score of 0.49 ± 0.08 compared to rural (15) that had an average aggression score of -0.40 ± 0.09.

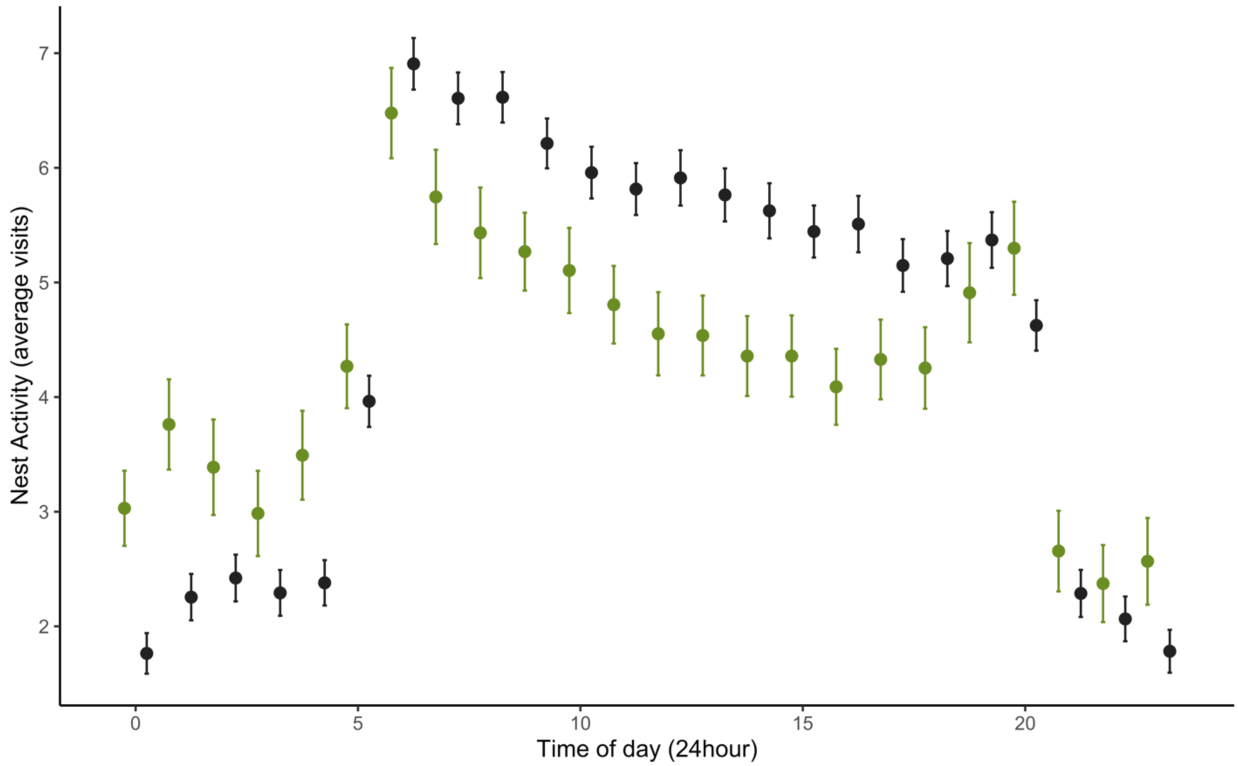

Figure 4: Maternal visitations varied between urban and rural (green) habitats during the nestling period (day 0 – 10) depending on time of day. Urban females appear to increase nest activity compared to rural during the day, and the opposite occurs at night. Urban females (24) visited the nest on average 5.84 ± 0.10 times/hour during nestling provisioning compared to rural females (16) that visited the nest on average 5.24 ± 0.16 times/hour. During these visits, urban females spent on average 41.94 ± 2.89 minutes per visits compared to rural, who spent on average 55.99 ± 4.76 minutes per visit. Each time point is averaged from 216 urban observation (days) and 67 rural observations (days).

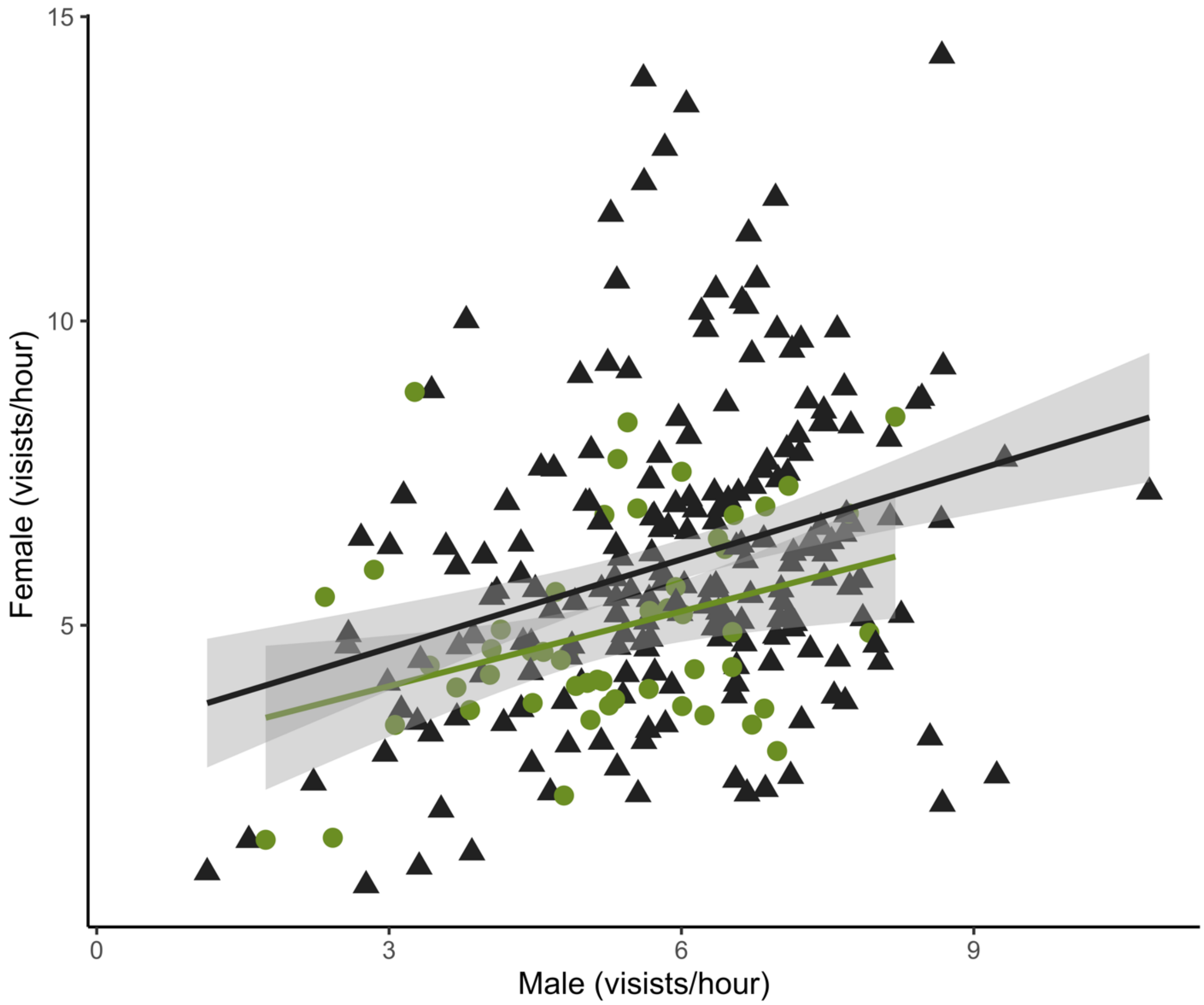

Figure 5: Social partner care was positively corelated during offspring provisioning in both urban (triangles) and rural (green circles) habitats. Females (41) that visited the nest on average 5.72 ± 0.10 times/hour had social partners (41) that visited on nest on average 5.80 ± 0.14 times/hour. Each data point represents the average care provided in a day by the female and her social partner for a total of 274 days of parental care data.

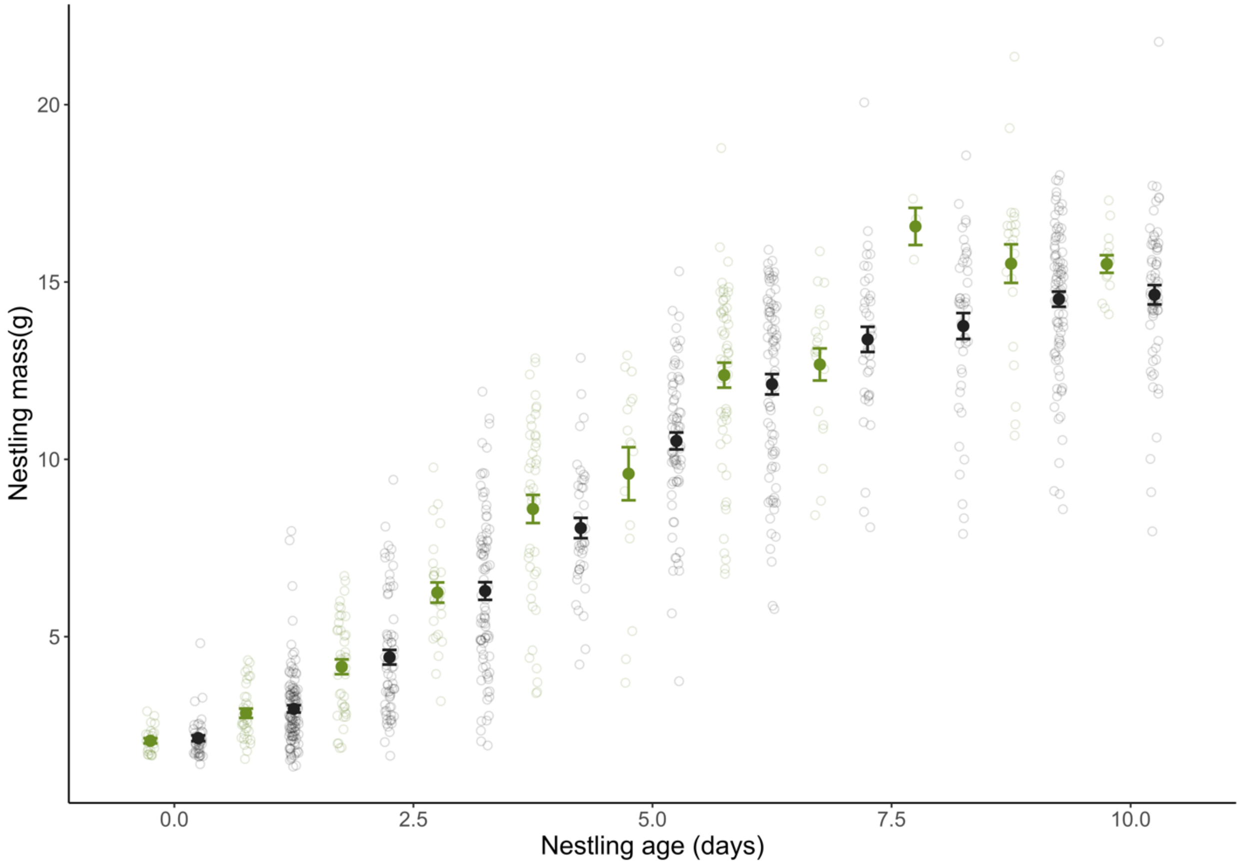

Figure 6: Song sparrow nestling growth between urban (349 nestlings) and rural (green; 128 nestlings) habitats. Urban nestlings were larger on average (9.06 ± 0.18) compared to rural nestlings (8.47 ± 0.29). However, urban nestlings had significantly slowed growth compared to rural nestlings. Means were average from 746 urban measurements (118 nests) and 302 rural measurements (47 nests).

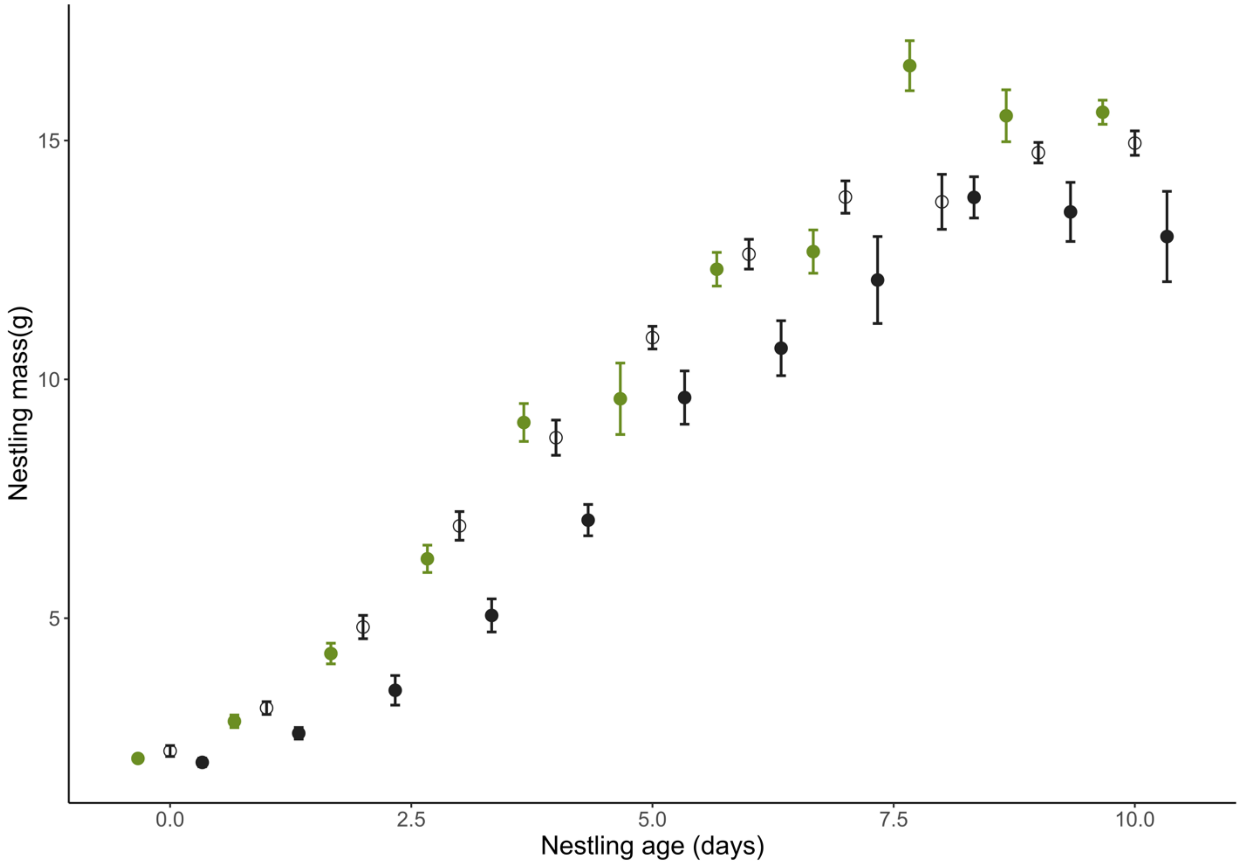

Figure 7: Nestling development between habitat types and in the presence of brood parasitism. Song sparrow nestling mass is significantly lower in nests containing brown-headed cowbirds compared to those without. Rural (green) nestlings (8.54 ± 0.29) and urban (open black) nestlings (9.64 ± 0.22) were larger on average without brown-headed cowbird nestlings, compared to urban (closed black) nestlings (7.65 ± 0.33) competing with brood parasite nestlings. Only 1 rural nest was document with cowbird nestlings and was removed from the data for this comparison.

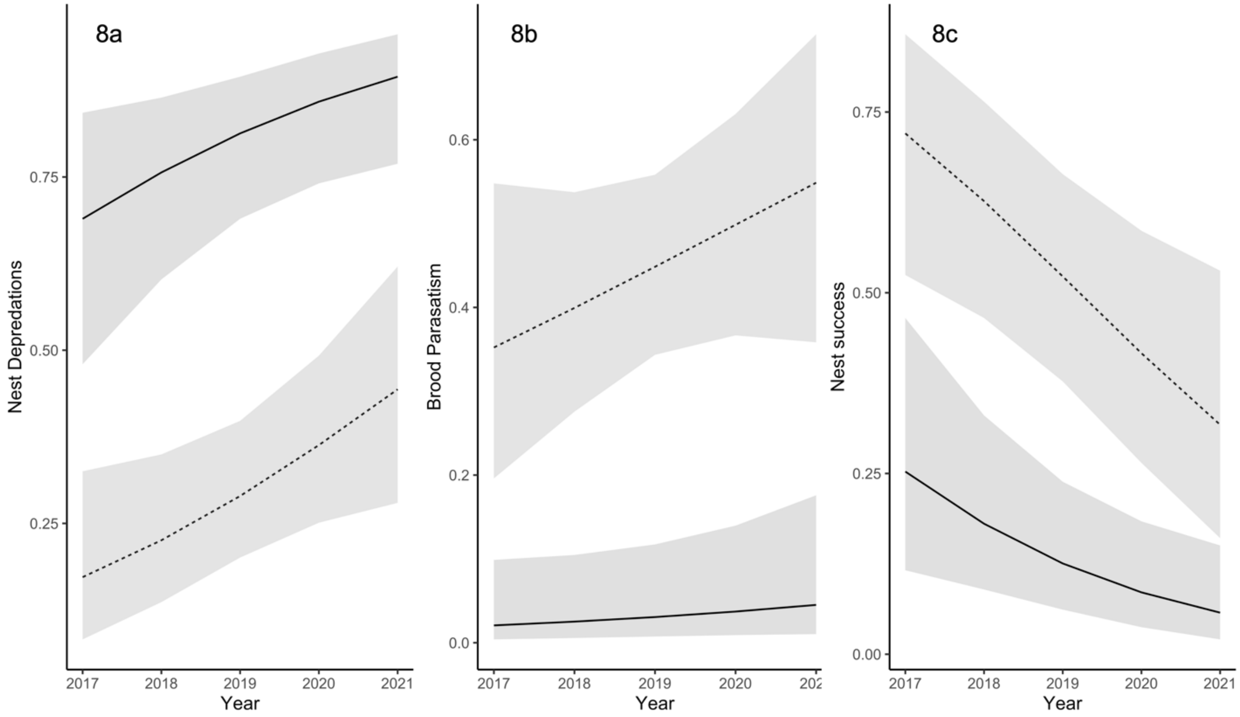

Figure 8: Urban (dotted line) and rural (solid line) probability of nest depredation (a) brood parasitism (b) and nest success c) pressure from 2017 - 2021. Urban (dotted, gray) habitats (166 nest) had significantly higher nest success and rates of brood parasitism across all 5 years compared to rural (119 nests; solid, green). However, nestling success in urban habitats decreased across years and rates of brood parasitism increased. Rural habitats saw significantly higher levels of nest predation across all years compared to urban, but urban nest predation rates increased across years.

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Appendix C: Chapter 3 Supplementary Materials

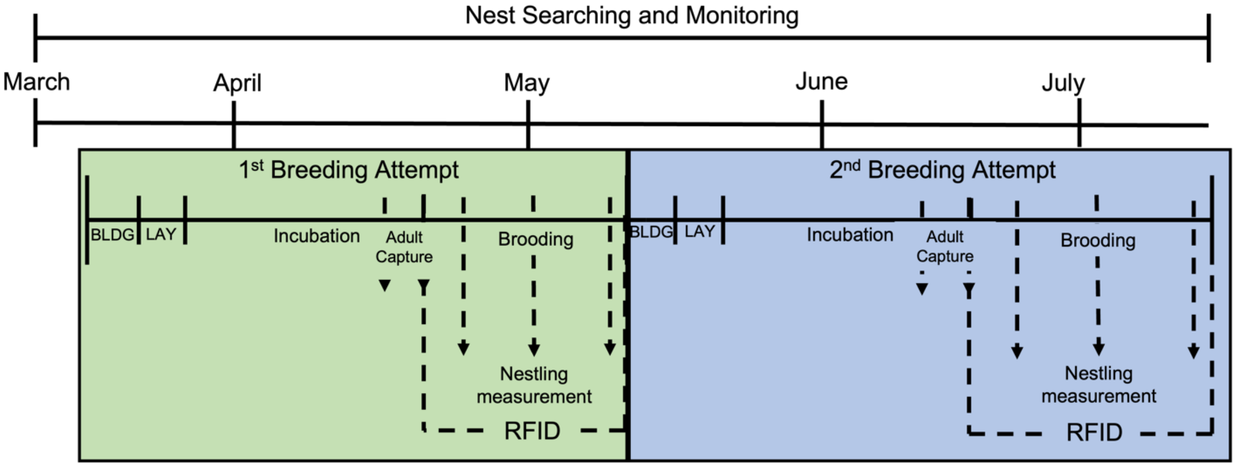

Figure C1: An example of a hypothetical timeline for methods conducted during the breeding season.

Table C1: Principal component analysis of male aggressive behaviors

Importance of components

	PC1	PC2	PC3
Standard deviation	1.2632	0.9006	0.7702
Proportion of Variance	0.5319	0.2704	0.1977
Cumulative Proportion	0.5319	0.8023	1

Dissertation Conclusion

My dissertation advances our knowledge of how songbird behavior and physiology is altered to acclimate to novel urban habitats, and the benefits of such flexibility. Specifically, my research presents evidence of neural and physiological habituation in urban males and informs our understating of enhanced territorial aggression in urban habitats, compared to rural. This pattern is well established in songbirds and other taxa, but my research adds to our understanding of this pattern by describing the potential costs and benefits associated with gaining an urban territory, which may in turn drive differences in behavior. This research has implications for the fundamental understanding of the effects of urbanization on life history trade-offs and neurobiology of urban birds. In the following paragraphs I detail further directions I suggest for each area of research covered in my dissertation.

Hypothalamic-pituitary-adrenal axis regulation and organization in urban and rural song sparrows.

Identifying consistent differences in physiology between urban and rural birds has been difficult, especially when considering circulating levels of corticosterone (Bonier 2012). Previous studies on our focal populations (Beck et al. 2018; Davies et al. 2018; Foltz et al., 2015), as well as recent review papers, have illustrated the discrepancy in circulating hormone results when considering the effects of urbanization on wildlife (Injaian et al. 2020). However, there are other aspects of the glucocorticoid stress response that could facilitate establishment and retention of urban songbird territories that remain unexplored. In Chapter 1, I provided evidence for physiological differences in the negative feedback system of the glucocorticoid stress response, as well as differences in circulating levels of corticosterone between urban and rural male songbirds. This variation in physiology could allow for rapid acclimation to urban environments, where individuals are presented with persistent novel stressors. Given the variability of the glucocorticoid stress response, an adrenocorticotropic challenge could show the physiological capacity for urban and rural birds to release glucocorticoids (Partecke et al. 2006). These results would begin to clarify whether the disparate results across labs in circulating cort between urban and rural songbirds are due to cognitive filters regarding stressors, or physiological differences in their capacity to release circulating hormones. Additionally, most studies investigating the effects of urbanization on wild bird physiology have focused on males (Bonier 2012; Caro 2012). Urban endocrine studies should consider different sexes and ages classes to gain a holistic understanding of the effects of urbanization on the physiology of wild birds (Caro 2012)

I find the 11β-HSD2 results especially exciting, as this enzyme is very understudied, especially in wild animal populations (Pérez 2020), and has great potential to serve not only as a protective mechanism, but to modulate the glucocorticoid stress response in urban habitat (Wyrwoll et al. 2011). Elevated levels of 11β-HSD2 in the hypothalamus have been shown to experimentally alter the strength of negative feedback of the glucocorticoid stress response (Harris et al. 2001; Pérez 2020). However, we still do not understand the relative importance of this enzyme when compared to other negative feedback mechanisms. Future studies that manipulate receptor availability and enzyme abundance through blocking of receptors and canulization of 11β-HSD2 could disentangle the two and illuminate their relative importance in regulating this response in urban habitats. This future work would not only enhance our understanding of the physiology of urban animals, but also our understanding of how the glucocorticoid stress response functions.

What about females? Urban female song sparrows elevate aggressive signaling compared to rural.

It has been consistently observed that males in urban habitats express different behavioral phenotypes compared to rural males (Davies and Sewall 2016; Evans et al. 2010; Ewers and Didham 2006; Foltz et al. 2015; Gomes et al. 2011; Hagan et al. 1996; McKinney 2002; Myers et al. 2016; Saunders et al. 1991). However, female behavior in urban ecology has been generally overlooked, except around maternal care (Heppner and Ouyang 2021). In Chapter 2, I provided evidence of further behavioral differences between urban and rural animals by showing that females song sparrows, like males, express elevated aggressive signaling towards conspecifics, though this behavior did not constrain their ability to provide maternal care. Future avenues of research should explore other aspects of female aggression, including their response to conspecific males, as females assist in territorial defense (Elekonich 2000; Wingfield 1994; Wingfield and Monk 1992). Additionally, female response to heterospecifics, such as brown-headed cowbirds, should be investigated as our understanding of these interactions is limited, and brood parasitism has great impact on host nest success.

We do know urban habitats increase the rates of brood parasitism (Chapter 3), therefore if urban female song sparrows can defend their nests against brood parasites, this increased heterospecific aggression could further increase their fitness. The presence of brood parasites is known to elicit aggressive responses from hosts (Ellison & Sealy 2007; Gloag et al 2013; Jelínek et al. 2021; Neudorf & Sealy 1994), and my research and other studies have shown that nest parasitism rates are higher in urban habitats (Burhans & Thompson 2006; Chace et al. 2003; Rodewald, 2009; Tewksbury et al. 2006). Birds breeding in areas of increased brood parasite presence, such as the brown-headed cowbirds in urban areas, will likely show increased aggression compared to areas of lower parasite density, and would likely facilitate resistance against brood parasitism of their nests. Future studies should compare the aggressive response of urban and rural female songbirds to the presence of brown-headed cowbirds to see if there is increased heterospecific aggression. Additionally, an aggressive response towards brood parasites is not always successful and can simply modulate the brood parasite's behavior to the host (Feeney et al. 2012; Jelínek et al. 2021). By comparing levels of aggression to the likelihood of brood parasitism, we could see if heterospecific aggression in urban habitats is an effective defense and could be advantageous.

Finally, across 6 years of study, I regularly observed females' territory tenure in urban habitats surpassing that of their social partner. Multiple females remained on their territories after their social partner had moved territories or died. For example, individual 277149465 held the same territory for 6 years, with 3 different males. She returned to nest in the same locations seemingly independent of her social partner. This female and females with similar behavioral phenotypes would cycle through multiple males during their tenure but would be specific about their nest site selection. In urban habitats, where nesting locations for open cup nesting songbirds are limited (Reale and Blair 2005; Seress and Liker 2015), females may be constrained in their choice of nesting locations and their ability to choose between males. Future studies that focus on specific territories, using removal experiments to track territorial establishment, and manipulations of male ornamentation that could enhance or reduce could disentangle the importance of female mate choice verses territory choice in urban and rural habitats.

Effects of urbanization and male aggression on parental care and life history outcomes in a wild songbird

Maximizing energetically costly behaviors during the breeding season often constrain the expression of other costly reproductive behaviors. In Chapter 3, I was unable to establish a trade-off between elevated aggression and parental care in males. In fact, more aggressive urban males visited the nest significantly more often than rural males, and birds in urban habitats had higher fledgling success, despite significantly higher rates of brood parasitism by brown-headed cowbirds. Collectively, these results suggest that urban song sparrows are coping effectively with urban challenges, and their physiology reflects a beneficial dampening of their stress response in a habitat with frequent stressors. Increased aggression is likely advantageous in this habitat, but it is unclear if there is pressure for males to be more aggressive (i.e., only the most aggressive males establish territories) or if the reduction in nest predation, possible changes in energy limitations (Goodchild et al. 2022) and altered male-male competition release males from limitations on aggression.

The use of RFID on open cup nesting species revealed an interesting relationship among females between urban and rural habitats. Though not statistically significant across a 24-hour period, urban females appear to visit the nest more often during the day, where rural females had high nest visitation rates at night. As offspring provisioning occurs during the day, these results suggest that nestling provisioning increases in urban environments. Seeing as daily nest visitation rate did not differ across the 24-hour period, urban and rural birds may have different daily time budgets, though it is difficult to speculate since we have little to no information about the nocturnal activity of open cup nesting songbirds. Given the differences in light-at-night between urban and rural habitats (Hopkins et al. 2018; Korpach et al. 2022), investigating the nocturnal time budgets of diurnal species could add insight into the daily energy requirements of urban and rural birds. These results also show the importance of considering time of day in measuring female behavior. Males express the same level of care across the day, but female behaviors vary significantly. Only looking at a snapshot of the day, or only considering a single sex in ecological or physiological studies could alter the interpretation of the results and ignore changes in behavior throughout the day (Caro 2013; Hau et al. 2017).

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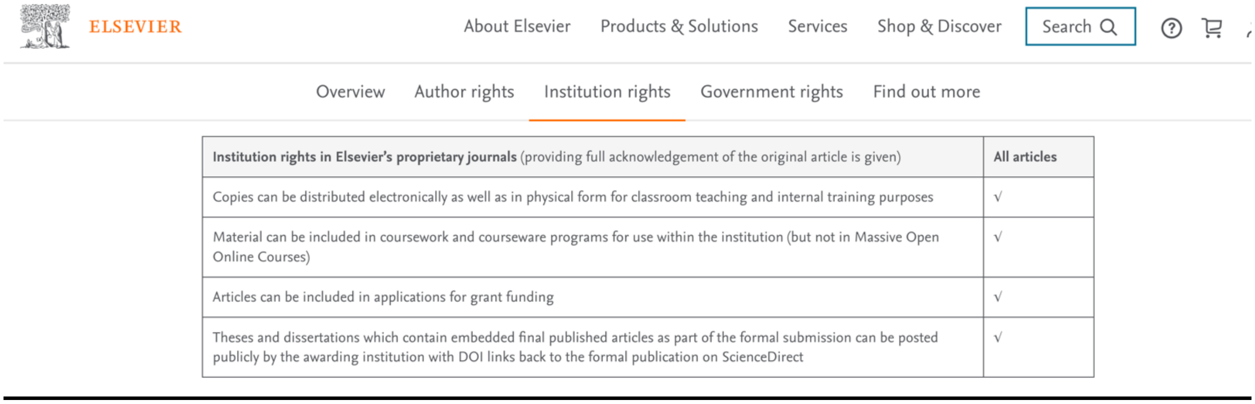

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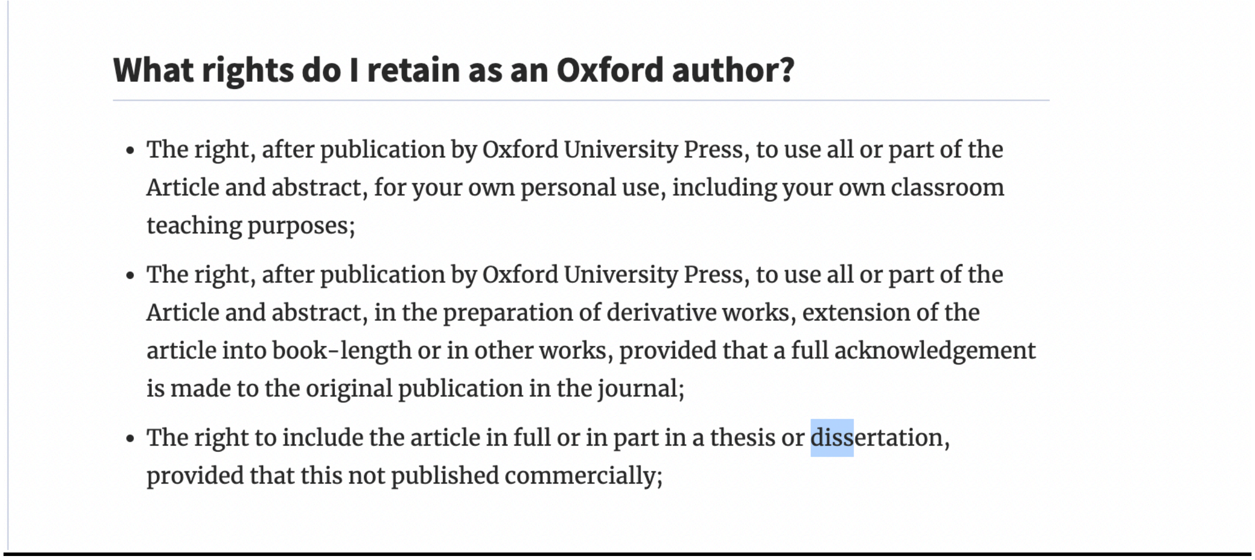

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