Effects of Human Disturbance on Physiology, Behavior, and Ornamentation in the Eastern Bluebird

Lauren Marjorie Gillespie
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EFFECTS OF HUMAN DISTURBANCE ON PHYSIOLOGY, BEHAVIOR, AND ORNAMENTATION IN THE EASTERN BLUEBIRD

by

Lauren Marjorie Gillespie

A Dissertation
Submitted to the Graduate School
and the Department of Biological Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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August 2016
ABSTRACT

EFFECTS OF HUMAN DISTURBANCE ON PHYSIOLOGY, BEHAVIOR, AND ORNAMENTATION IN THE EASTERN BLUEBIRD

by Lauren Marjorie Gillespie

August 2016

Overall, few studies have focused on anthropogenic disturbance on wildlife physiology. Research has typically focused on how environmentally contaminated areas or anthropogenic disturbance (e.g. noise, human activity) influences biodiversity, community structure and behavior of individual animals. However, understanding how disturbance influences some aspects of physiology can require sacrifice of the animal, prohibiting ecologically relevant measures of behavior and reproductive success. This research strives to examine covariation between testosterone (T) and corticosterone (CORT), plumage ornamentation, and behavior in two populations of eastern bluebirds (Sialia sialis) that differ in degree to which their habitat is modified by human activity.

In this research I compare bluebirds breeding in a suburban golf course with those breeding at a rural site exposed to lower levels of human disturbance. I demonstrate that golf course females lay eggs later, produce smaller clutch sizes, and golf course pairs fledge fewer offspring. Males at the golf course population respond to live, conspecific intrusion with lower T levels and less aggression, and are more highly ornamented compared to a more rural population. Moreover, within the golf course site, but not the rural site, I found that males show an increase in T from nest building to incubation. Females at the
golf course respond to live, conspecific intrusion with higher raw T and less aggression, and are more highly ornamented than the rural population. Females from both sites increase T and CORT from nest building incubation, however, aggression is not correlated with T. Results imply that golf course females cannot elevate T and display behavioral aggression. Lastly, golf course pairs take longer to complete nests and display increased nest attendance while golf course females only provision nestlings at significantly higher rates. Results here demonstrate support for the hypothesis that level of human disturbance subtly impacts behavior and physiology in bluebirds. This research adds to a growing body of literature stressing the importance of investigating physiological measures of disturbance in wild populations that are experiencing realistic selection pressures.
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DEDICATION

I cannot thank my family enough for their support throughout my graduate career. I thank both of my parents for choosing to raise me in rural Maine and for constantly challenging me. I thank my mother for introducing me to nature and wildlife and my father for convincing me that I can accomplish anything. I thank my sister for being the guiding compass of our family and maid of honor at my defense. I thank both she and her husband for my two amazing nephews, Tripp and Mason, whose smiling faces brightened many a day in the process of the writing of this document. I thank my godfather’s, Robert and James Melaugh, for providing invaluable guidance in every arena that exists.

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CHAPTER I - ENDOCRINOLOGY, BEHAVIOR, AND DISTURBANCE:
SUBTLE ENVIRONMENTAL DIFFERENCES BETWEEN TWO HUMAN-
MODIFIED SONGBIRD BREEDING HABITATS

Introduction

In the mid-twentieth century, eastern bluebird (Sialia sialis) populations declined due to habitat loss and reduction in nesting resources. Bluebirds build nests and raise young within cavities, but cannot self-excavate, relying upon previously excavated and then abandoned cavities or naturally formed cavities for breeding (Gowaty and Plissner, 1998). Backyard birders found that by providing nest boxes, bluebirds would readily breed in them, facilitating population increases (Gowaty and Plissner, 1998). Bluebirds prefer to nest in habitats consisting of open, short grass areas with a forest edge (preferably containing snags of dead wood) (Gowaty and Plissner, 1998; Jones et al. 2014) which can be limited in human-dominant environments. Providing nest boxes for bluebirds in urbanized areas preferentially relocates them to suburban backyards, cemeteries, agricultural lands, state parks, military bases, university campuses, and golf courses. Human-modified environments of this nature often vary drastically in stimuli considered disturbing to wildlife (e.g. levels of both human activity and land-use practices), potentially eliciting differential physiological or behavioral responses based on extent of disturbance (reviewed in Tablado and Jenni, 2015).

Physiological correlates to anthropogenic disturbance are important for understanding species-specific adaptations to a changing environment.
Experimental design regarding human disturbance is evolving to examine fitness-relevant measures of physiology and behavior in wild populations. Such measures are reviewed here and discussed in detail in later chapters as they pertain to bluebirds. Relative measures of disturbance and habitat characteristics from study sites are presented in this chapter along with reviews of information relevant to the behavior and physiological states to be investigated in this dissertation. This chapter provides justification for classifying study sites by ‘level’ of high or low disturbance. Here, I review information related to human disturbance and the hormones, behaviors, and ornaments that might be impacted by this disturbance as increased noise and anthropogenic disturbance has been shown to alter self-maintenance behavior, productivity, and song in bluebirds (Kight and Swaddle, 2007; Kight et al. 2012; Kight and Swaddle, 2015).

**Human Disturbance**

Anthropogenic impacts on wildlife fitness are more intensive when responses elicited in wildlife are chronic or more intense themselves, and, such responses can vary based on species niche and context or timing of disturbance events (reviewed in Tablado and Jenni, 2015). Areas with lower levels of human disturbance have higher species richness and abundance (Kang et al. 2015). In birds, human activities negatively affect hatching rate (González et al. 2006) and lower nest survival probability (Westmoreland and Best, 1985). Research shows that birds, and other wildlife, may perceive humans as predators (reviewed in Frid and Dill, 2002; reviewed in Tablado and Jenni, 2015). This forms the basis for the risk-disturbance hypothesis, which predicts that individuals will invest
more energy towards anti-predator strategies (e.g. increases in vigilance or aggression) in response to increased interaction with disturbing stimuli, resulting in reduced reproductive success (reviewed in Frid and Dill, 2002). This hypothesis will be explored in other chapters in relation to behavior (reviewed in Clotfelter et al. 2004), steroid hormones (reviewed in Gore and Dickerson, 2012), and ornamental plumages (Carere et al. 2010), all shown to be affected by disturbance.

*Steroid Hormones*

Steroid hormones examined in this research, testosterone (T) and corticosterone (CORT), are involved in regulatory and physiological mechanisms associated with behavior. Testosterone is involved in mediating aggression, mating, reproductive, and parental care behaviors (reviewed in Adkins-Regan, 2005) while CORT mediates metabolic energy reserves and stress responses (reviewed in Carsia and Harvey, 2000). Production of T (gonads) and CORT (adrenal glands) begins with cholesterol, and both are made through a number of enzymatic conversions. Steroids are rarely stored, necessitating their creation de novo in response to stimuli (reviewed in Adkins-Regan, 2005). Once produced, androgens may be converted to estrogens via aromatase to exert biological actions (reviewed in Adkins-Regan, 2005). Steroids, which are lipophilic molecules, move easily through tissues and cell membranes, eventually affecting gene transcription and resulting in production of proteins (reviewed in Gore and Dickerson, 2012). Both T and CORT have peripheral and neural target tissues
through which they mediate their effects on physiology and behavior (reviewed in Gore and Dickerson, 2012).

To produce T and CORT, the hypothalamus releases excitatory hormones in response to stimuli. Corticotropin-releasing hormone (CRH) and gonadotropin-releasing hormone (GnRH) reach the anterior pituitary stimulating the release of adrenocorticotropic hormone (ACTH) and luteinizing hormone (LH); these, in turn, stimulate production of CORT and T, respectively. Both endpoint hormone classes are regulated by negative feedback loops sustaining hormone levels within homeostatic ranges (reviewed in Adkins-Regan, 2005; reviewed in Gore and Dickerson, 2012; Carsia and Harvey, 2000). The structure of each of these hormones is highly conserved across species; this allows for non-human animals, such as birds, to serve as model organisms for mammals, including humans, as they are endothermic and metabolize substances similar to humans (reviewed in Adkins-Regan, 2005; reviewed in Gore and Dickerson, 2012; Carsia and Harvey, 2000).

*Testosterone in Males.* Androgens, specifically T, are typically higher in males than females and are involved in permissive and inhibitive actions regarding aggressive behavior, mating and reproduction, and parental care. Aggression is utilized to compete for and/or defend territories, resources, and mates (reviewed in Adkins-Regan 2005). Exogenous implants of T have illuminated aspects of male social behavior under influence of increased levels of T (Wingfield et al. 1987; reviewed in Ketterson et al. 2009). Birds treated with the anti-androgens (for example, flutamide) exhibit decreases in aggression
(reviewed in Ketterson et al. 2009; Sperry et al. 2010), further supporting an androgen dependent response for aggressive behaviors. Males with higher levels of T often have increased reproductive success (reviewed in Møller et al. 2005) but this can come at the cost of reduced body condition and survival (reviewed in Adkins-Regan, 2005). In male songbirds, the normal annual profile for T involves higher levels in early breeding when aggressive and reproductive interactions occur, and lower levels throughout parental care stages, particularly in bi-parental species (Wingfield, 1984). Individual variation in male aggression can be predicted by circulating hormones in some species (Peterson et al. 2013) but not in others (DeVries et al. 2012). Additionally, gene expression for other androgens (e.g. 5α-dihydrotestosterone, androstendione) and androgen receptors, estrogen (E2) receptors, and aromatase may predict variation in male aggression (Rosvall et al. 2012).

**Testosterone in Females.** Gene expression for T, E2 receptors and aromatase also predicts aggression in females (Rosvall et al. 2012). Similar to males, circulating T has been the focus of investigation in female aggression, however unlike males, circulating T levels infrequently correlate with aggression (Rosvall et al. 2012). Selection for high levels of T in males may cause increased selection pressure on females as T concentrations in female passerine birds are often positively correlated to that of males (reviewed in Ketterson et al. 2005; reviewed in Møller et al. 2005; reviewed in Ketterson et al. 2009; reviewed in Goymann and Wingfield, 2014). Investigations of behavioral and mechanistic facilitation of female aggression have reflected similar methodology as studies
involving males (reviewed in Rosvall, 2013). However, as the sexes experience different selection pressures, the notion that circulating T mediates aggression similarly in males and females is under reconsideration (reviewed in Ketterson et al. 2009; reviewed in Goymann and Wingfield, 2014). By implanting female dark-eyed juncos with T, researchers show similar behavioral results as in males, such as increased aggression and decreased parental care (Zysling et al. 2006; Cain and Ketterson, 2012). More recent studies show differential gene expression of both males and females exposed to implanted T suggesting that lower levels of T in females may facilitate aggression (Peterson et al. 2013).

Differential mechanistic facilitation of female aggression (especially in relation to steroid hormones) is of interest in songbirds due to well documented ‘maternal effects’, a term describing relationships between organizational and/or activational effects of yolk steroid hormones, offspring genotype, and environmental factors influencing nestling survival (reviewed in von Engelhardt and Groothuis, 2011). In many species, females have lower T than males, and potentially low circulating female T is an adaptive strategy to maximize offspring fitness by avoiding damaging offspring impacts (e.g. elevated offspring mortality) that have seen with elevated T and maternal effects (reviewed in Groothuis and Schwabl, 2008; von Engelhardt and Groothuis, 2011).

_Corticosterone._ Stress is generally defined as any experience that disrupts the body’s homeostatic state. Daily functions require glucocorticoids (GCs; cortisol in mammals, CORT in birds) to access energy stores and therefore GC are always present in the blood (e.g. predictive homeostasis, baseline levels,
(see Romero, 2004 for full model description). In situations in which the brain receives notice that the current homeostatic state has been altered, the animal undergoes a stress response also mediated by GCs; levels of GCs in this situation may drastically increase (reactive homeostasis, stress-induced levels, (see Romero, 2004)). Epinephrine is released initially during a stress response, resulting in increases in heart rate, blood pressure, awareness, visual acuity, hearing, and memory, accompanied by decreased pain perception as well as redirection of blood flow to limbs and other essential organs (reviewed in Kuenzel, 2000). These effects are short-lived, and if the stressor continues, the stress response is then maintained by GCs, making glucose available (e.g. gluconeogenesis in liver, among other functions) and redirecting energy towards necessary functions survival (reviewed in Carsia and Harvey, 2000). If the stressor is prolonged, the response becomes maladaptive, and GCs may accumulate which can induce myopathy in muscles and temporarily or permanently suppress reproduction (reviewed in Breuner, 2011). If or when the stressor subsides, GCs then serve as recovery hormones helping to regain homeostasis (Romero, 2004). Concentrations of GCs measured from blood or other tissues can be used to demonstrate effects of environmental perturbation on individuals and populations (reviewed in Sol et al. 2013). Organisms ideally are examined for both baseline and stress induced GC levels, as levels of GCs in these situations have different physiological and behavioral endpoints (Romero, 2004).
Corticosterone in Adults. Corticosterone and T facilitate similar physiological and (sometimes) behavioral responses to conspecific aggression (reviewed in Adkins-Regan, 2005; reviewed in Carsia and Harvey, 2000). While this is an aggressive interaction, an agonistic challenge for territory ownership which elicits a T response is also an inherently stressful situation leading to elevations in GCs. Reactions between androgens and GCs are not unexpected, and are frequently observed in implant studies (Schoech et al. 1999). Additionally, environmental disturbance and reduced individual condition due to living in marginal habitat can also impact stress responses in birds (Wingfield et al. 1983; Beale and Monahan, 2004; Cockrem, 2013).

Corticosterone in Nestlings. Environmental differences may influence stress responses in nestlings of many species resulting in varied CORT responses early in life and variation in adult behavior later (Blas et al. 2005; Mayne et al. 2004; Franceschini et al. 2008). Nestling CORT has been found to vary between nestlings within the same broods, suggesting that stress caused by competition in the nest is widespread (Saino et al. 2003; Eraud et al. 2008). Indeed, experiments show that nestlings that experience food stress display increased CORT (Saino et al. 2003; Honarmand et al. 2010). Nest environment can have direct effects on nestling CORT and behavior in eastern bluebirds, as laying order predicts hatch order (Gowaty and Plissner, 1998). Later hatched bluebird nestlings beg more and weigh less, however, they do not display higher CORT than earlier hatched siblings (Soley et al. 2011). Bluebird nestlings from larger broods display higher plasma T, and CORT is higher in males when
broods are cross-fostered, suggesting a strong impact of nest environment (Kozlowski and Ricklefs, 2011). House sparrows (*Passer domesticus*) nestlings with experimentally elevated CORT beg more, are fed less by parents, and display reduced growth rate and immune response (Loiseau et al. 2007). These studies demonstrate individual variation in CORT of nestlings, with impacts on behavior later in life, leading to variation in hormone levels in adults and ultimately affecting fitness.

*Ornamental Plumages*

There are three main types of ornamental plumage coloration used in avian signaling: carotenoid and melanin pigments and structural coloration (reviewed in McGraw, 2006). Most yellow, orange or red color is produced by carotenoid pigments while most grey, rufous, brown, and black colors are produced by melanin pigments (reviewed in McGraw, 2006). Ultraviolet (UV), blue, green, purple and iridescent coloration is produced by feather nano- or micro-structure (reviewed in Prum, 2006). Plumages examined here are ultraviolet (UV) blue structural coloration and melanin pigmented coloration. Both plumages have the potential to honestly indicate body condition and can be affected by nutrient access (McGraw et al. 2002) and environmental stress (reviewed in Hill, 2006). Melanin and structural coloration are also thought to be genetically influenced with stronger empirical evidence for melanin coloration (reviewed in Mundy, 2006).

*Structural Coloration.* Structural colors in avian feathers are produced through coherent scattering of light by nanostructures of the feather barbule.
Arrangements of beta-keratin granules and size of air cavities or channels in medullary cells in the epidermis determine color, and when openings are larger, a longer wavelength of color is observed (reviewed in Prum, 2006). These plumages typically function as reliable indicators of mate quality or competitive ability (reviewed in Griffith and Pryke, 2006). High quality UV blue ornaments correlate with increased body mass and territory size of male blue grosbeaks \textit{(Guiraca caerulea)} (Keyser and Hill, 2000). Female bluethroats \textit{(Luscinia svecica)} that display more colorful ornamental throat-patches display increases in body mass and tarsus length, and males prefer more colorful females as mates (Amundsen et al. 1997). In blue tits \textit{(Parus caeruleus ultramarinus)}, males are aggressive towards decoys with experimentally dulled UV reflectance, (Alonso-Alvarez et al. 2004) while male bluebirds are more likely to attack decoys with experimentally brighter, more chromatic UV blue coloration (Mercadante and Hill, 2014). Both studies suggest a role for structural coloration in male-male interactions.

\textit{Melanin Pigmentation}. Melanin pigments in birds consist of the eumelanins (dark black, dark brown) and the phaeomelanins (reddish-brown, chestnut, rufous) and both pigments are synthesized from amino acids tyrosine and cysteine, respectively (reviewed in McGraw, 2006). Both amino acids are can be made \textit{de novo} or obtained from diet. Eumelanins and phaeomelanins molecules differ in size, shape, and composition, while differing amounts of each pigment influence color perceived (reviewed in McGraw, 2006). Synthesis occurs in epidermal melanocytes and pigments are deposited into feathers and feather
tracts. High levels of tyrosinase drive eumelanin synthesis whereas lower levels produce phaeomelanins (reviewed in McGraw, 2006), and steroid hormones can alter tyrosinase activity (reviewed in Kimball, 2006). Melanin pigments serve a number of adaptive functions (reviewed in McGraw, 2006), protecting against both oxidative damage and heavy metal accumulation in the body; they can also be indicative of physiological states, signal mate quality, or honestly predict dominance relationships (see reviews in Jawor and Breitwisch, 2003; McGraw 2006, Roulin, 2015).

What constitutes a high quality melanin ornament and how melanin ornament quality is described can be confusing. When measuring melanin ornaments, light reflectance, ‘presence of white’, or brightness, is what is measured (reviewed in McGraw, 2006). A ‘brighter’ bird, in relation to a melanin pigmented ornament, is a less ornamented bird, meaning the feather reflects more white, and therefore less pigment is present. Darker melanin pigments are considered to be of higher ornament quality as more pigment is present in the feather, reflecting less light (reviewed in McGraw, 2006). High quality ornaments in male house sparrows (darker melanin pigmented breast bibs) indicate both dominance and body condition (Veiga, 1993; Nakagawa et al. 2007; Nelson, 2011). Male house sparrows are less likely to instigate and win aggressive interactions with males possessing larger bibs (Liker and Barta, 2001), demonstrating efficient and honest signaling; additionally, males of this species implanted with T develop larger bibs, and are more effective in acquiring and defending resources (Evans et al. 2000; Strasser and Schwabl, 2004). Lastly, in
this species, condition dependence of melanin ornaments is impacted by deficiencies in amino acids phenylalanine and tyrosine (rate limiting for melanin synthesis), causing production of brighter, less ornamented, plumage (Poston et al. 2005).

*Ornamental Plumages in Bluebirds*

Ultra-violet blue feathers of male and female bluebirds differ in nanostructure, particularly in the diameter of circular air spaces and cortex levels of feather barbs (Shawkey et al. 2005). Female females have thicker cortices, likely causing lower UV reflectance compared to males (Shawkey et al. 2005). Male eastern bluebirds with brighter, more brilliant UV blue coloration and darker melanin pigmented breast patches achieve higher reproductive success in both Alabama and Oklahoma populations (Siefferman et al. 2005b; Grindstaff et al. 2012). Ultra-violet plumage increases with male age in the Alabama population (Siefferman et al. 2005). These plumage ornaments may signal parental effort (Siefferman and Hill, 2005a) or indicate hormone titers (Grindstaff et al. 2012; Siefferman et al. 2013), which may signal aspects of mate quality. This plumage may also signal dominance among males (Mercandante and Hill, 2014). Given the intricate relationships between ornamental plumages, physiology, and behavior, they may be useful in indicating contaminant or disturbance exposures in birds (Carere et al. 2010).

*Site Selection*

Bluebird nesting ecology and tolerance of general territory disturbance make them an ideal study species for investigations of disturbance on behavior
and physiology. Bluebirds willingly nest in human-provided boxes (Gowaty and Plissner, 1998), which allows researchers to choose the study environment. To study effects of disturbance on bluebird physiology and behavior, I elected to do a comparison study of populations exposed to different levels of disturbance (high versus low).

A golf course represented the high disturbance site with the assumption of high human activity. This allowed for fitness-relevant measures of physiology to be taken in the light of high anthropomorphic disturbance. Previous research performed on bluebirds breeding on golf courses show delayed first egg dates in one population (Stanback and Seifert, 2005) while a second population displayed increased egg and fledgling production but lower pre-hatch nest survival (LeClerc et al. 2005). A rural military based represented the low disturbance site, with the assumption of infrequent human-bluebird interactions given the size and operative nature of the base. Land-use practices of this site consisted of annual grass maintenance via mowing (Chris Pontin, Camp Shelby Environment Office, pers comm.). There appears to be geographical variation for correlative relationships between ornamental plumages, physiology, and behavior among bluebirds (Lynn Siefferman, pers. comm.), implying these phenotypes may be flexible in their expression depending on environmental circumstances.

**General Site Differences**

For this dissertation research, eastern bluebird populations at a golf course and a rural military base in Mississippi are examined for subtle impacts of disturbance on physiology and behavior. Sites were selected due to observed
differences in disturbance. Most habitats available to, and preferred by, bluebirds are modified by humans to some extent. An examination of different levels of modification allows for investigation of more realistic disturbance exposures. Here, I examine and report on environmental differences between the sites serving as a reference for the remainder of this dissertation.

The golf course (high disturbance, 31 20.9' N, 89 22.6' W, Hattiesburg MS) is located approximately 29.77 (km) kilometers from Camp Shelby Joint Forces Training Center (hereafter ‘Camp Shelby’, low disturbance, 31.1878° N, 89.1992° W, Hattiesburg, MS) (Figure 1). Golf carts, maintenance vehicles, and people travel the golf course from 0500-1900 daily. Human activity occurs year-round (estimated 20,000 rounds of golf initiated annually, Tom Ricks, pers. comm.) but increases markedly in May, June, and July, coinciding with peak breeding season of bluebirds present on site (L.M. Gillespie, pers. obs.). This site also utilizes daily, seasonal, and annual grounds maintenance through both mowing and use of pesticides (for full product detail, see Table 1). Camp Shelby is a National Guard training base, with prescribed vehicle and human activities occurring annually at the same time and for the same duration (highest activity is in late July into August as birds are finishing breeding). There are over 250 boxes on the Camp Shelby site but only 120 were monitored; these boxes are located in areas experiencing infrequent human traffic and annual grounds maintenance (mowing only; Chris Potin, pers. comm.; L.M. Gillespie, pers. obs.).
Table 1

Products in Use at Golf Course

<table>
<thead>
<tr>
<th>Brand</th>
<th>Active Ingredient (Chemical Class)</th>
<th>Mechanism of Action</th>
<th>Target Pest</th>
<th>TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barricade</td>
<td>Prodiamine (Dinitroaniline)</td>
<td>Inhibits microtubule formation, mitotic process (US DOE, 2006)</td>
<td>Grasses, weeds</td>
<td>III</td>
</tr>
<tr>
<td>Specticle</td>
<td>Indaziflam (Alkylazine)</td>
<td>Inhibit cellulose biosynthesis (Brabham et al. 2014)</td>
<td>Grasses, weeds</td>
<td>III</td>
</tr>
<tr>
<td>Trimec</td>
<td>2,4-Dichlorophenoxyacetic Acid (2,4-D) (Phenoxyacetic Acid)</td>
<td>Increased cell-wall plasticity, ethylene production; uncontrolled cell division (Gervais et al. 2008)</td>
<td>Weeds</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td>Trimec, Celsius</td>
<td>Mecoprop or (MCPP) (Chlorinated phenoxy)</td>
<td>Synthetic auxin; systemic</td>
<td>Weeds</td>
<td>III</td>
</tr>
<tr>
<td>Trimec</td>
<td>Dicamba (Benzoic acid)</td>
<td>Rapid/uncontrolled cell division/growth (US EPA, 2006)</td>
<td>Weeds, woody plants</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV</td>
</tr>
<tr>
<td>Roundup</td>
<td>Glyphostate (Phosphonoglycine)</td>
<td>Inhibits enzyme-substrate complex (EPSP) (USDA, 1999; Schönbrunn et al. 2001)</td>
<td>Weeds</td>
<td>III</td>
</tr>
<tr>
<td>Certainty</td>
<td>Sulfosulfuron (Sulfonylurea)</td>
<td>Inhibits amino acid synthesis (CA DPR, 2008)</td>
<td>Weeds, grasses</td>
<td>III</td>
</tr>
<tr>
<td>Metsulfuron-Methyl</td>
<td>Methyl benzoate (Sulfonylurea)</td>
<td>Inhibits amino acid synthesis (WA, DOT, 2006)</td>
<td>Weeds, trees</td>
<td>III</td>
</tr>
<tr>
<td>Monument</td>
<td>Trifloxsulfuron-sodium (Sulfonylurea)</td>
<td>Inhibits amino acid synthesis (US EPA, 2008)</td>
<td>Grasses, NA sedges,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>weeds</td>
<td>NA</td>
</tr>
<tr>
<td>Celsius</td>
<td>Iodosulfuron (Sulfonylurea)</td>
<td>Inhibits amino acid synthesis</td>
<td>Grass, weeds</td>
<td>NA</td>
</tr>
</tbody>
</table>
Table 1 (continued).

<table>
<thead>
<tr>
<th>Brand</th>
<th>Active Ingredient (Chemical Class)</th>
<th>Mechanism of Action</th>
<th>Target Pest</th>
<th>TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merit</td>
<td>Imidaclorod (Neonicotinoid)</td>
<td>Post-synaptic disabling of nicotinic acetylcholine receptors (Johnson et al. 2010)</td>
<td>Insects, fleas, mites</td>
<td>II</td>
</tr>
<tr>
<td>Talstar</td>
<td>Bifenthrin (Pyrethroid)</td>
<td>Nervous system modulation (delay sodium ion channel closure); may inhibit ATP (Johnson et al. 2010)</td>
<td>Insects</td>
<td>II</td>
</tr>
<tr>
<td>Fipronil</td>
<td>(±)-5-amino-1-(2,6-dichloro-α,α,α-trifluoro-p-tolyl)4-trifluoromethylsulfinylpyrazole-3-carbonitrile (Phenylpyrazole)</td>
<td>Blocks GABA-gated chloride channels; affects binding affinity (Jackson et al. 2009)</td>
<td>Arthropods</td>
<td>II</td>
</tr>
<tr>
<td>Daconil</td>
<td>Chlorothalonil (Chloronitrile)</td>
<td>Prevents spore germination/motility (Kegley et al. 2014)</td>
<td>Broad spectrum fungicide</td>
<td>II</td>
</tr>
<tr>
<td>Derivative</td>
<td>Acibenzolar-S-methyl (Benzothiadiazole)</td>
<td>Induces plant defenses (Kegley et al. 2014)</td>
<td>Mildew, White rust, Bacteria</td>
<td>NA</td>
</tr>
<tr>
<td>Briskway</td>
<td>Difenoconazole (Triazole)</td>
<td>Inhibits demethylation &amp; sterol biosynthesis, alters membrane permeability</td>
<td>Broad spectrum fungicide</td>
<td>III</td>
</tr>
<tr>
<td>Briskway, Heritage</td>
<td>Azoxystrobin (Strobilurin)</td>
<td>Inhibits mitochondrial respiration; inhibits spore production/germination (Ministry of Agriculture and Lands, 2009)</td>
<td>Rusts, mildew, White mould</td>
<td>NA</td>
</tr>
<tr>
<td>Torque</td>
<td>Tebuconazole (Triazole)</td>
<td>Inhibits demethylation &amp; sterol biosynthesis, alters membrane permeability (Kegley et al. 2014)</td>
<td>Smut, Bunt</td>
<td>III</td>
</tr>
<tr>
<td>Banner</td>
<td>Propiconazole (Triazole)</td>
<td>Inhibits demethylation &amp; sterol biosynthesis, alters membrane permeability (Kegley et al. 2014)</td>
<td>Broad spectrum fungicide</td>
<td>II</td>
</tr>
<tr>
<td>Mancozeb</td>
<td>Maneb (Carbamate)</td>
<td>Inhibits respiration, enzyme and amino acid synthesis</td>
<td>Broad spectrum fungicide</td>
<td>IV</td>
</tr>
</tbody>
</table>
Table 1 (continued).

<table>
<thead>
<tr>
<th>Brand</th>
<th>Active Ingredient</th>
<th>Mechanism of Action</th>
<th>Target Pest</th>
<th>TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mancozeb</td>
<td>Zineb (Carbamate)</td>
<td>Inhibit respiration, enzyme and amino acid synthesis</td>
<td>Broad spectrum fungicide</td>
<td>IV</td>
</tr>
</tbody>
</table>

List (Tom Ricks, pers. comm). TC= Environmental Protection Agency’s Toxicity Categories based on acute oral (category I: 50 mg/kg-category IV: >5000 mg/kg), dermal (category I: 200 mg/kg-category IV: >5000 mg/kg) and inhalation (category I: 0.05 mg/liter-category IV: >2 mg/liter) effects and primary eye (category I: corrosive- category IV: minimal) and skin (category I: dermis destruction- category IV: mild irritation) irritation ranging (US EPA, 2014). All located in database for those with sources not listed in table (University of Hertfordshire, 2007); additional sources listed in table.

Sites were specifically analyzed for relative measures of disturbance, habitat characteristics, and nest box density to quantitatively demonstrate and account for environmental differences within and between sites. These characteristics are important to quantify when studying bluebirds as behavior and reproductive performance can be impacted by habitat type and box density (Bhardwaj et al. 2015; Jones et al. 2014). Here, I compare 1) measures of human presence, golf carts, and field maintenance vehicles, 2) habitat variability, and 3) nest box densities between the golf course and Camp Shelby to fully justify the labeling of these environments as high and low disturbance.

**Methods**

**Collection of Relative Measures of Disturbance:**

During nestling feeding observations (see Chapter V for full description), either myself or field assistants recorded the number of humans on foot (both sites), number of cars (Camp Shelby) or golf carts (golf course), and the number
of military (Camp Shelby) or grounds maintenance vehicles (both) during hour long nestling provisioning observation sessions. Most nests received two observations, although some only received one, and number of observations did not impact results. Boxes experiencing human or vehicle traffic uncharacteristic to site descriptions (e.g. Camp Shelby boxes located on main roadsides) were discarded from analyses for both relative measures of disturbance and individuals at those boxes were eliminated from subsequent analyses in future chapters as these boxes were located in areas uncharacteristic of the specific site in general.

Habitat Characterization and Nest Box Density Calculation

Study sites were located approximately 29.77 km apart. GPS locations of boxes at the golf course were collected using a handheld Garmin GPS (model: ETREX 20) while GPS locations of boxes at Camp Shelby were provided by their environmental office. Analysis of habitat was performed according to Jones et al. (2014) in conjunction with John Jones (Appalachian State) Briefly, October 2014 National Agriculture Imagery Program (NAIP) imagery obtained from EarthExplorer (earthexplorer.usgs.gov) was used to classify habitat structure surrounding eastern bluebird nest boxes (following methods presented in Jones et al. in press). NAIP imagery is georeferenced and is available at a 1m resolution. One image for northern field sites (golf course, Figure 2) and four adjacent NAIP images were used to classify the southern field sites (Camp Shelby, Figure 3). NAIP images were classified using an Interactive Supervised Classification in ArcMAP 10.3 (ESRI, Redlands, CA, USA), generating four
habitat characteristics: (1) Abiotic structures (abiotic factors made by humans; e.g., roads, buildings); (2) open habitat (i.e., hypothetically suitable foraging area; Gowaty and Plissner, 1998; Jones et al. 2014); (3) Forested/Canopy cover; and (4) Water. Next, Extract by Mask tool in ArcMAP 10.3 was used to calculate the percent cover of each habitat characteristic for a 75m buffer (as average territory size is between 50-100 m) surrounding the nest box. Buffers paired with occupancy records (2013-2015) allowed for calculation of distance to nearest occupied nest box in meters (m), which will be used as a measure of density and hereby referred to as ‘box density’.

Results

Habitat and Nest Box Density

ANOVA results for site comparisons of relative measures of disturbance, habitat characteristics, and nest box density are shown in Tables 2 and 3. The golf course has significantly more human traffic, a higher percent of open habitat and water, lower percent abiotic structures, and boxes are more densely located. There are no differences between sites in percent forest cover (Table 2, Figure 2). Last, the golf course nest boxes are significantly more densely located than Camp Shelby nest boxes (Table 2, Figures 2 and 3).

Table 2

Measures of Disturbance, Habitat, and Nest Box Density

<table>
<thead>
<tr>
<th>Variable</th>
<th>$F_{(df)}$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Human Traffic</td>
<td>25.62$_{(1,61)}$</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Average Car/Golf Cart Traffic</td>
<td>9.21$_{(1,62)}$</td>
<td>0.004</td>
</tr>
</tbody>
</table>
Table 2 (continued)

<table>
<thead>
<tr>
<th>Variable</th>
<th>$F_{(df)}$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Field Maintenance or Military Vehicle Traffic</td>
<td>0.863(1,59)</td>
<td>0.357</td>
</tr>
<tr>
<td>Water</td>
<td>34.783(1,151)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Abiotic Structure</td>
<td>45.151(1,160)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Open Habitat</td>
<td>18.380(1,160)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Forest Cover</td>
<td>0.307(1,160)</td>
<td>0.580</td>
</tr>
<tr>
<td>Density</td>
<td>91.351(1,76)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

ANOVA results for site comparisons of relative measures of disturbance, habitat characteristics, and nest box density.

Habitat characteristics measurements are percentages.

Table 3

Mean Values: Disturbance Measures, Habitat, and Density

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Traffic</td>
<td>1.37</td>
<td>2.30</td>
<td>0.47</td>
<td>0.17</td>
</tr>
<tr>
<td>Car/Golf Cart Traffic</td>
<td>3.15</td>
<td>5.38</td>
<td>9.08</td>
<td>9.17</td>
</tr>
<tr>
<td>Field Maintenance and Military Vehicles</td>
<td>1.37</td>
<td>2.45</td>
<td>1.56</td>
<td>2.96</td>
</tr>
<tr>
<td>Water</td>
<td>2%</td>
<td>0.05</td>
<td>0.3%</td>
<td>0.004</td>
</tr>
<tr>
<td>Abiotic Structure</td>
<td>6%</td>
<td>0.05</td>
<td>18%</td>
<td>0.13</td>
</tr>
<tr>
<td>Open Habitat</td>
<td>40%</td>
<td>0.12</td>
<td>28%</td>
<td>0.18</td>
</tr>
<tr>
<td>Forest Cover</td>
<td>52%</td>
<td>0.12</td>
<td>54%</td>
<td>0.22</td>
</tr>
<tr>
<td>Density</td>
<td>126.69m</td>
<td>18.13</td>
<td>340.31m</td>
<td>128.89</td>
</tr>
</tbody>
</table>

Mean values are for raw data; relative disturbance measures are counts of humans and motor vehicles; habitat characteristics are percentages; density measures are average distance to nearest occupied nest box in meters.
Figure 1. Study Sites in Hattiesburg, MS

Golf course located in upper left corner and Camp Shelby in lower right corner. Dots represent 75m buffers surrounding boxes.

Figure 2. Golf Course in Hattiesburg, MS

Dots represent 75m buffers surrounding boxes.
Figure 3. Camp Shelby, Hattiesburg, MS

Dots represent 75m buffers surrounding boxes.

Relative Disturbance

Nest boxes at the golf course experience significantly higher human foot traffic compared to Camp Shelby (Table 2, Figure 4) while Camp Shelby nest boxes experience significantly higher car traffic (smaller vehicles) than golf course nest boxes (here, golf carts). There were no differences between sites in occurrences of larger vehicles (e.g., field maintenance vehicles at the golf course or military vehicles at Camp Shelby) during observations.
Figure 4. Average Humans during Nestling Feeding

Humans Passing Nest Box = number of humans passing nest boxes during 1 hour observation periods. The line in the box is the median, the boxes are the 25th and 75th percentiles, the whiskers are the 10th and 90th percentiles, and the stars are outliers.
Sites examined in this dissertation research differ significantly in relative measures and types of disturbance, habitat characteristics, and nest box density. Golf course nest boxes experience significantly higher human foot traffic passing during nestling feeding as predicted (Table 2, Figure 4). Contrary to predictions, golf course nest boxes experience significantly less light traffic vehicles (golf carts) passing while nest boxes at Camp Shelby experience increased light traffic vehicles (cars) passing during nestling feeding (Table 2, Figure 5). There is no
difference between sites in frequency of larger vehicles (field maintenance vehicles at the golf course; military vehicles at Camp Shelby) passing by nest boxes, also contrary to original predictions. The most prominent difference between sites is the occurrence of people outside of vehicles near nest boxes at the golf course. This may disturb breeding bluebird more than fast moving vehicles, as prolonged leisurely golfer activity may increase bird stress, and research shows humans can be perceived as predators (Frid and Dill, 2002).

Habitat characteristics differed significantly between sites. The golf course has a higher percent open habitat than Camp Shelby, as predicted; the golf course also has higher percent water and lower percent abiotic structures (e.g. roads and buildings) than Camp Shelby (Table 1, Figure 2, Figure 3). There are no differences between sites in percent forest cover.

**Conclusions**

Statistical tests and general site observations demonstrate that the designation of the golf course as the high disturbance site and Camp Shelby as the low disturbance site is justified. Sites also differ in grounds maintenance practices with the golf course experiencing year-round, daily maintenance of grounds (mechanical care and pesticide application) and Camp Shelby having once-yearly mowing and no pesticide treatments. Grounds care routines may amplify the disturbance from human presence at the golf course and potentially adult or nestling food availability or quality could be influenced by these activities.

Quantitatively, compared to Camp Shelby, golf course birds experience chronic, unpredictable and significantly increased human traffic near boxes daily,
with peaks in activity correlating with peaks in breeding. Birds at Camp Shelby experience significantly higher numbers of motor vehicles passing during feeding observations. While this result is statistically significant, it does not appear to visibly bother birds (L.M. Gillespie, pers. obs; Jodie Jawor, pers. obs.). While cart traffic at the golf course may not be statistically higher, birds are visibly and vocally distressed when people or carts pass or spend time close to occupied nest boxes (L.M. Gillespie, pers. obs). For example, behavioral observations were often abandoned on tournament days at the golf course, when both human and golf cart presence is greatly increased, as the birds themselves abandoned feeding attempts, sometimes for hours, during tournaments (L.M. Gillespie, pers. obs). Eastern bluebirds were readily present at both of the study areas and have been in residence at both places for a number of years, demonstrating that individuals are drawn to them, however, what the variation in disturbance means ecologically and behaviorally to individuals inhabiting these sites is not well understood.

It is important to explore physiological correlates of quantifiable relative disturbance measures and habitat characteristics when investigating anthropogenically disturbed populations. Disturbance of this nature is widespread, encompassing many levels of influence possibly impacting physiology. This allows for unique behavioral and physiological assessment, while accounting for selection pressures present in the wild. This is the approach this research takes; following chapters will examine differences in ornamental plumages between sites compared to aggression, parental care behaviors, and
hormone titers in both sexes. Disturbance exposures are not limited to one life history stage, and therefore examination of physiology and behavior throughout the breeding cycle and in both nestlings and adults will be investigated, as certain developmental periods are more sensitive to alterations in behavior and physiology (reviewed in Gore and Dickerson, 2012).
Birds, particularly males, are renowned for a diversity of plumage colors and such traits are used in species recognition, dominance interactions and female mate choice (reviewed in Bortolotti, 2006; reviewed in Hill, 2006). If ornamental plumage coloration is sexually selected, highly ornamented males are predicted to demonstrate higher competitive ability, provide better genes, or higher quality parental care (reviewed in Griffith & Pryke 2006). Elaboration of male plumage is influenced by costs associated with producing, maintaining, or displaying ornaments (reviewed in Hill, 2006).

In many species of birds, both sexes are monochromatically colored while, in other species, sexes are dimorphic in color (reviewed in Bortolotti, 2006). Sexually dimorphic species often show drastically different plumage colors between males and females; typically, female coloration is similar, but less conspicuous than male coloration (reviewed in Amundsen and Pärn, 2006). Two explanatory hypotheses for females expressing duller versions of male ornaments are debated, with empirical evidence to support both. Similarities are hypothesized to result from high genetic correlation between the sexes or sexual selection acting directly on females as it does in males (reviewed in Amundsen and Pärn, 2006; reviewed in Hill, 2015; reviewed in Siefferman and Hill, 2005a).
Direct selection on plumage traits signaling aspects of quality in females does occur in some species (reviewed in Amundsen and Pärn, 2006; reviewed in Clutton-Brock, 2007; reviewed in Tobias et al. 2012). Female ornaments often correlate with aggression associated with resource or territory defense (reviewed in Amundsen and Pärn, 2006) while male ornaments often signal fighting prowess, parental care efforts, or reproductive success (reviewed in Hill, 2006).

*Ornamental Plumages*

Most plumage coloration in birds are carotenoid-based (reviewed in McGraw, 2006), melanin-pigment-based (reviewed in McGraw, 2006), or structurally-based (reviewed in Prum, 2006). Focus here will be on melanin pigment and structural UV blue color which are both biologically produced via different means (see Chapter I for full detail). Past research on both melanin-based and structurally-based plumages shows potential for condition dependence as both are influenced by nutritional status or access to certain nutrients (McGraw et al., 2002). Additionally, both maternally derived and exogenous hormones (reviewed in Kimball, 2006), environmental stress (reviewed in Hill, 2006), and genetics (reviewed in Mundy, 2006) may all influence production, maintenance, or physiological capacity to display these ornaments.

*Melanin Pigments.* Melanin pigments are produced in melanocytes and deposited into the feather barbule following other physiologically orchestrated and carefully timed cascades of cellular events (reviewed in McGraw, 2006). The
melanins consist of the eumelanic pigments (dark blacks and browns) and the phaeomelanic pigments (reddish rufous and chestnut varieties) (reviewed in McGraw 2006). Both pigments require amino acids for production; higher levels of tyrosine typically yield eumelanins while higher levels of cysteine produce predominantly phaeomelanins (reviewed in McGraw, 2006). Most melanin-based plumage coloration results from differing ratios of each pigment type (reviewed in McGraw, 2006). Eumelanins are more frequently studied with respect to ornamentation than phaeomelanic pigments as these are more frequently associated with cryptic colorations (variable within individuals) (reviewed in Bortolotti, 2006; reviewed in McGraw, 2006). A high quality melanin ornament is a darker ornament, containing more pigment, whereas a lower quality melanin ornament, or a ‘brighter’ ornament, reflects more white and contains less pigment overall (reviewed in McGraw, 2006; see Chapter I).

*Ultra-violet Structural Coloration.* The coherent scattering of light via nanostructural elements of the feather barb (composed of arrangements of circular beta-keratin rods interspersed with air pockets; see Chapter I for full detail) yields non-iridescent colors including UV reflectance (reviewed in Prum, 2006; Shawkey et al. 2005). In eastern bluebirds (*Sialia sialis*), keratin rod number predicts UV chroma, and increased number of uniformly sized keratin rods equates to expression of higher, more ornamented UV chroma (Shawkey et al. 2003; Shawkey et al. 2006).
Ornamentation in Bluebirds. In eastern bluebirds, there are well-established relationships between ornamental plumages and behavior (reviewed in Siefferman and Hill, 2005a, 2005b). More ornamented male eastern bluebirds express brighter, UV blue coloration on the back, wings, and tail and a darker melanin pigmented breast patch. In males ornaments indicate behavior and individual quality. For example males expressing more ornamented UV color are more successful in acquiring limited nest boxes (Siefferman and Hill, 2005b). More-ornamented males (both greater UV blue chroma and darker melanin breast color) pair with females that breed earlier in the season, provision young more often, and fledge larger young (Siefferman and Hill, 2005a; Grindstaff et al. 2012). Bluebird UV blue plumage is condition dependent (Siefferman and Hill, 2008; Doyle and Siefferman, 2014), increases with age (Siefferman et al. 2005), and UV color between the sexes differs structurally and quantitatively (Shawkey et al. 2006). Males displaying less ornamented breast color exhibit higher T (Siefferman et al. 2013) while males and females with brighter UV color have higher CORT and lower T (Grindstaff et al. 2012). Higher quality UV ornaments may indicate physical or attack prowess, as increases in UV signals (of models) elicit more aggressive responses from males (Mercadante and Hill, 2014). Bluebird males also tradeoff physiological resources between UV color and parental care, with increases in paternal investment impacting both future UV ornament quality and timing of reproductive effort (Siefferman and Hill, 2005c).
Lastly, UV blue coloration of nestlings influences provisioning rates of parents, for example males feed brighter sons more (Ligon and Hill, 2010a, 2010b).

*Bluebird Ornaments as Indicators of Anthropogenic Disturbance*

Many cavity nesting species such as tree swallows (*Tachycineta bicolor*) (Dakin et al. 2016), great tits (*Parus major*) (Remeš and Matysioková, 2013), and eastern bluebirds (reviewed in Siefferman and Hill, 2005a, 2005b) experience strong selection pressures based on limited nesting resources and have well documented relationships between both ornaments of interest and behavior or physiology. These species are great candidates for study of ornamental plumages, as they utilize a life history strategy providing several advantages for ease of study, particularly that of species willingly and successfully breeding in human dominated environments, as is the case with bluebirds. Historic bluebird population numbers decreased due to limitations in available breeding cavities, and human-provided nest boxes now aid once limited populations (Gowaty and Plissner, 1998). Bluebirds display preferences for open, short grass environments interspersed with trees and snags (Gowaty and Plissner, 1998; Jones et al. 2014) and habitats of this nature can be limited in urban landscapes. Provided nest boxes brought bluebirds into human-modified and disturbed areas, potentially exposing birds to selection pressures different from those present in a more natural environment.

As ornamental plumages can be costly and correlate with physiology, they are becoming more accepted as physiological indicators of disturbance (Carere
et al. 2010), and, as such, were chosen for analysis in this dissertation. Environmental stress (natural and anthropogenic) can result in reduced physiological capability to produce energetically expensive ornaments (reviewed in Hill, 2006). Additionally, alterations in hormone titers of testosterone (T) or corticosterone (CORT) can result in altered melanin or UV blue plumage ornamentation (Evans et al. 2000; Strasser and Schwabl, 2004; Roulin et al. 2008; Siefferman et al. 2013; reviewed in Roulin 2015). Coloration of melanin-pigmented and UV blue chroma are examined in two populations of eastern bluebirds, one residing at a golf course and another at a rural military base. Sites (described in Chapter I) represent the high and low end of disturbance exposures as many breeding birds now reproduce in areas with some degree of human modification. My hypothesis is that level of disturbance will subtly impact plumage ornaments of male and female bluebirds. I predict that golf course males and females will display reduced expression of both ornaments (lower UV tail chroma and increased, lighter melanin breast color).

Methods

Study Sites

*High Disturbance Location.* A golf course (Hattiesburg, MS) served as the site for representing high levels of human disturbance. The turf is maintained daily and kept short through mowing and pesticides are applied frequently; there is a higher percent of open habitat (preferable bluebird habitat) and boxes are more densely located (see Chapter I). I monitored 45 boxes at this site.
Low Disturbance Location. Camp Shelby Joint Forces Training Center ([CSJFTC] Hattiesburg, MS; hereafter Camp Shelby) served as the low disturbance site. There is little to no grounds maintenance in areas where boxes were monitored (mowing biannually); there is lower percent open habitat, higher percent buildings and roads, and boxes are less densely located (see Chapter I). I monitored ~120/250 boxes on site.

Study Species

Bluebirds begin prospecting for territories and mates in February and March and may have 2-3 clutches (mean 4-5 eggs) until breeding ceases in August in Southern populations. Due to nesting and foraging requirements of bluebirds, their exposure to humans, and the fact that not all human habitats have the same level of disturbance, this species is ideal for this study.

Bird Capture

All capture attempts occurred from 0400-1200h weather permitting. Birds were captured in mist nets or box traps, standard morphometric measures were taken (weight, tarsus length, wing chord, tail length) and they were equipped with a USGS identification band (permit #23479) and unique combination of color bands. The golf course received a preliminary season of analysis in 2012 from May-August. During 2013 and 2014, the golf course and Camp Shelby, respectively, were monitored February through August, and during 2015, both sites were surveyed simultaneously from February through June.
Plumage Analysis

After capture, I collected plumage samples from the breast (I removed a small portion of feathers from the body with surgical scissors) and tail (I plucked the two outer-most tail feathers). Using a low reflectance black paper (Canson® Drawing Paper, Stygian Black #425), I individually stacked 7-10 breast feathers upon one another as they would appear on the bird and taped tail feathers (at feather base) on either side of breast plumage.

Reflectance spectrometry is commonly used to measure avian coloration and was utilized in this research (reviewed in Montgomerie, 2006). Ultra-violet plumage was measured on a spectrometer (OceanOptics S2000; range 250-880nm; Dunedin, FL) tuned toward the UV range (ultra-violet, blue, violet) and melanin pigmented plumage was measured on a spectrometer tuned toward the visual spectrum (violet-red). Utilizing spectrometers geared towards specific wavelengths allows for more accurate reflectance, especially for melanin pigments which fall within the visible spectrum (reviewed in Andersson and Prager, 2006). Color variables analyzed were UV chroma and melanin brightness; UV chroma is a measure of the spectral purity of a color (sometimes called saturation), or the percentage of light reflected in the UV range (reviewed in Montgomerie, 2006). Brightness is a measure of the percentage of white light reflected from a feather (amount of white present), or a measure of ‘lightness’, and this terminology can be confusing (reviewed in Montgomerie, 2006). The word ‘brightness’ is often associated with more brilliant color perception,
however, for melanin pigments, a brighter bird is a ‘lighter’ bird, with plumage containing less pigment and therefore is considered less ornamented (reviewed in Montgomerie, 2006).

Melanin brightness was calculated using a color reflectance spectrophotometer (OceanOptics S2000; range 300-700nm; light source: tungsten/deuterium) and SpecraSuite software. I used a micron fiber probe (90 degree angle) touching the feather surface until the spectral curve appeared on the screen, and then saved measures. For each sample the number of reflectance measures taken between wavelengths 300-700nm (the area under the curve) were averaged yielding a whole number measure of brightness (% reflectance) ranging from 0-35.

Tail feathers were stored in light-proof boxes until being sent to Dr. Lynn Siefferman (Appalachian State University, Boone, NC) for UV plumage analyses. Protocols for spectral analyses of feathers were performed following methods outlined in Siefferman et al. (2005) and Siefferman and Hill (2007). Using a micron fiber probe held at a 90 degree angle 2mm from feather surface, three replicates of measurements from each feather sample for all birds were taken and averaged. For UV blue structural coloration, mean UV chroma was calculated as the proportion of total light reflectance in the UV spectrum (300-400nm) (reviewed in Prum, 2006), yielding a decimal number measure of UV chroma (% reflectance) ranging from 0.225-0.345.
Statistics

SPSS (version 23.0) was utilized for analysis. All data were tested for normalcy using Shapiro-Wilk and most variables were not normally distributed (P < 0.05). Spearman’s correlations were used within sites to determine bias in collection date. I standardized reflectance measures by collection date for all years (accounting for temporal variation) (Table 1; both melanin brightness and UV chroma) using a linear regression with percent reflectance as the dependent variable and collection date as the independent variable; I then saved standardized residuals and used these data in future analyses. Within each site, ANOVA was used to determine annual variation and independent samples t-tests were used for site comparisons. A two-way ANOVA with independent variables of sex and site and dependent variables as melanin and UV reflectance measures was used to examine the extent of male and female ornamentation dimorphism between sites. Results from data collection at both sites in the same year (2015) and those from all study years combined (2012-2015) are presented as both reveal meaningful information on patterns of ornament difference between sites. Multiple comparisons were used (to correlate traits within sites) and to control or false discovery rates, a stepwise, Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) was used; the false discovery rate was set to 10% (McDonald, 2014) and the procedure sequentially reduces the number of comparisons.
Results

Annual and Collection Date Variation for Ornaments

Date of plumage collection was not significantly correlated with either UV or melanin ornament in either sex within the golf course. At Camp Shelby, females caught later in the season (those breeding later) expressed significantly brighter (less ornamented) melanin pigmentation (Table 4) and females caught later in the season express significantly greater UV tail chroma (more ornamented; Table 4). Also at Camp Shelby, males caught later in the season (those breeding later) expressed both significantly lower UV tail chroma and significantly brighter melanin pigmentation (lighter color) (Table 4). Both golf course and Camp Shelby males and females (Figures 6 through 9) display significant differences in melanin brightness and UV tail chroma between years (Table 5), indicating that ornaments examined are not stable among or within study populations, and variable factors within sites may differentially influence color production. Additionally, it appears that year affects melanin color expression more strongly than UV (Table 5, Figures 6 through 9) however the overall trend in melanin is for a darker coloration at the golf course (Table 7, Figure 6, Figure 9).
### Table 4

*Collection Date Variation*

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Melanin Brightness</th>
<th>UV Blue Tail Chroma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golf Course</td>
<td>0.14 (83)</td>
<td>-0.14 (199)</td>
</tr>
<tr>
<td>Camp Shelby</td>
<td>0.43*** (70)</td>
<td>0.24* (68)</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golf Course</td>
<td>-0.11 (75)</td>
<td>0.17 (86)</td>
</tr>
<tr>
<td>Camp Shelby</td>
<td>0.61*** (74)</td>
<td>-0.51*** (77)</td>
</tr>
</tbody>
</table>

Numbers shown are correlation coefficients (Rs); numbers in parentheses are sample sizes; *= p<0.05, **=p<0.001, ***=p<0.0001; *=significant after Benjamini-Hochburg correction procedure to control for multiple comparisons.

### Table 5

*Annual Ornament Variation*

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Year</td>
<td>N</td>
</tr>
<tr>
<td>Male</td>
<td>Melanin</td>
<td>2012</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>UV</td>
<td>2012</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>35</td>
</tr>
<tr>
<td>Female</td>
<td>Melanin</td>
<td>2012</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>UV</td>
<td>2012</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>36</td>
</tr>
</tbody>
</table>

Residualized values correcting for date of capture were used in these analyses. N=sample size; $F_{(df)}$= ANOVA test statistic; *= significant after Benjamini-Hochburg correction procedure to control for multiple comparisons.
Figure 6. Annual Variation in Male Melanin Ornament

There is significant variation annually in male melanin ornament within sites; raw data is used in graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For melanin ornaments, a lower number equates to a more ornamented bird.
Figure 7. Annual Variation in Male UV Chroma

There is significant annual variation in male UV chroma within sites; raw data is used in the graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For UV chroma, a higher number equates to a higher quality ornament.
**Figure 8. Annual Variation in Female Melanin Ornament**

There is significant variation annually in female melanin ornament within sites; raw data is used in the graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For melanin ornaments, a lower number equates to a more ornamented bird.

**Figure 9. Annual Variation in Female UV Chroma**

There is significant variation annually in female UV tail chroma within sites; raw data is used in the graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For UV chroma, a higher number equates to a higher quality ornament.

**Site Differences in 2015 Ornaments**

In 2015, golf course males had significantly lower UV tail chroma than Camp Shelby males (Table 6, Figure 7) while golf course females had slightly greater (though not significant) UV tail chroma than Camp Shelby females (Table 6, Figure 9). For both sexes, there were no significant differences between sites in melanin coloration for birds sampled in 2015 only (Table 6, Figure 6, Figure 8).
Samples collected in 2015 are only those from the earliest breeding birds, which, in other Southern populations of bluebirds studied, are usually birds with darker melanin breasts and those expressing brighter and more chromatic UV blue plumage (Siefferman et al. 2005); this is consistent with 2015 results (Figure 10, Figure 11), as well as early season results from plumage samples collected in 2013 at the golf course and in 2014 at Camp Shelby.

Table 6

2015 Site Differences in Ornamentation

<table>
<thead>
<tr>
<th></th>
<th>Golf Course</th>
<th>Camp Shelby</th>
<th>Independent Samples t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanin</td>
<td>-0.36</td>
<td>0.50</td>
<td>27</td>
</tr>
<tr>
<td>UV</td>
<td>-0.62</td>
<td>0.61</td>
<td>26</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanin</td>
<td>-0.88</td>
<td>0.41</td>
<td>22</td>
</tr>
<tr>
<td>UV</td>
<td>0.19</td>
<td>0.73</td>
<td>35</td>
</tr>
</tbody>
</table>

M= mean; SD= standard deviation; n=sample size; t= test statistic; df= degrees of freedom; *= significant after Benjamini-Hochburg correction procedure to control for multiple comparisons. Means and SD presented are for residualized values correcting for date of capture (used in these analyses).
**Figure 10. 2015 UV Chroma**

2015 measures of UV Chroma for both sexes; raw data is depicted in graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For UV chroma, a higher number equates to a higher quality ornament.
Figure 11. 2015 Melanin Ornament

2015 measures of melanin brightness for both sexes; raw data is depicted in graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For melanin ornaments, a lower number equates to a more ornamented bird.

Site Differences in Ornamentation for All Years

Both males and females at the golf course expressed significantly darker melanin color and significantly more chromatic UV tail color than Camp Shelby males and females (Table 7, Figure 12, Figure 13).

Table 7

Site Differences in Ornaments: All Years

<table>
<thead>
<tr>
<th></th>
<th>Golf Course</th>
<th>Camp Shelby</th>
<th>Independent Samples t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Melanin</td>
<td>-0.03</td>
<td>0.79</td>
<td>83</td>
</tr>
<tr>
<td>UV</td>
<td>-0.35</td>
<td>0.73</td>
<td>89</td>
</tr>
</tbody>
</table>
Table 7 (continued).

<table>
<thead>
<tr>
<th></th>
<th><strong>Golf Course</strong></th>
<th><strong>Camp Shelby</strong></th>
<th><strong>Independent Samples t-test</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M</em></td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>Melanin</td>
<td>-0.77</td>
<td>0.73</td>
<td>75</td>
</tr>
<tr>
<td>UV</td>
<td>0.76</td>
<td>0.88</td>
<td>86</td>
</tr>
</tbody>
</table>

M= mean; SD= standard deviation; n=sample size; t= test statistic; df= degrees of freedom; *= significant after Benjamini-Hochburg correction procedure to control for multiple comparisons. Means and SD presented are for residualized values correcting for date of capture (used in these analyses).

Figure 12. Melanin Ornament between Sites

Comparisons of melanin brightness for both sexes for all study years combined; raw data is depicted in graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For melanin ornaments, a lower number equates to a more ornamented bird.
Figure 13. UV Chroma between Sites

Comparisons of UV chroma for both sexes for all study years combined; raw data is depicted in graph. The line in the box is the median, the boxes are the 25th and 75th percentiles, and whiskers are the 10th and 90th percentiles. The circles are past the 10th and 90th percentiles. For UV chroma, a higher number equates to a higher quality ornament.

Site Differences in Magnitude of Sexual Dimorphism:

In both 2015 and for all years, there was a significant site by sex interaction suggesting the degree of sexual dichromatism varied with site. At the golf course, males and females displayed more similar UV tail chroma. At Camp Shelby, males and females displayed more divergent UV tail chroma (Table 8, Figure 13).
Table 8

Male and Female Ornament Dimorphism

<table>
<thead>
<tr>
<th>Ornament</th>
<th>2015</th>
<th>Variable</th>
<th>$F_{(df)}$</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanin</td>
<td>Site</td>
<td>0.208(1,104)</td>
<td>0.649</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>48.91(1,104)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Site*Sex</td>
<td>2.55(1,104)</td>
<td>0.114</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>Site</td>
<td>0.018(1,130)</td>
<td>0.813</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>96.29(1,130)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Site*Sex</td>
<td>7.9(1,130)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>All Years</td>
<td>Melanin</td>
<td>Site</td>
<td>72.76(1,301)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sex</td>
<td>54.29(1,301)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site*Sex</td>
<td>0.060(1,301)</td>
<td>0.806</td>
</tr>
<tr>
<td></td>
<td>UV</td>
<td>Site</td>
<td>23.19(1,319)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sex</td>
<td>82.61(1,319)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Site*Sex</td>
<td>6.23(1,319)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Residualized values correcting for date of capture were used in these analyses. N=sample size; $F_{(df)}=\text{ANOVA test statistic}$; *= significant after Benjamini-Hochburg correction procedure to control for multiple comparisons.

Discussion

Results Summary

For samples collected over the entire study period, both males and females at the golf course expressed significantly darker breast coloration and significantly greater UV tail chroma compared to birds at Camp Shelby (Figure 12, Figure 13, Table 7). Additionally, sexual dichromatism was less pronounced at the golf course but only for UV tail chroma. Adult return rates at the golf course are ~39% after 1 year (2012-2013) and ~9% after two years (2013-2015; golf
course was not surveyed in 2014), indicating that golf course habitat is not only
drawing more ornamented individuals in but retaining these individuals in
consecutive breeding seasons at a higher percentage than at Camp Shelby

In 2015, data showed no significant dichromatism in breast ornamentation
at either the golf course or at Camp Shelby (Table 6, Figure 11). In 2015,
however, site influenced UV chroma of tails, with golf course males showing
significantly lower UV chroma and golf course females express slightly greater
UV chroma compared to Camp Shelby birds (Table 6, Figure 10).

For all samples analyzed over the study period, there is a significant
correlation between date of sample collection and plumage color for breast color
for males and females at Camp Shelby only, with birds caught earlier in the
season expressing significantly darker breast plumage. Within sites, for both
sexes, there is also significant variation between years. At the golf course, male
and female breast color was darker in 2012 and 2015 than in 2013; however, the
overall trend is for a darker melanin coloration in golf course birds compared to
Camp Shelby birds. Within sites, for both sexes, UV chroma also varies
significantly with year, although year differences for UV ornamentation appear
less pronounced than breast coloration, indicating that this ornament may be less
sensitive to varying environmental factors impacting color production.

2015 Study Results
The only year in which there are no effects due to larger yearly variation in resources between the two sites or in the environment in general is 2015, when birds were surveyed at both sites simultaneously. However, I only collected data until May, not throughout August as with the 2013 (golf course only) and 2014 (Camp Shelby only) seasons. The 2015 samples of both plumage and hormones are for earlier nesting birds and first nests of pairs from both sites. Earlier breeding bluebirds are traditionally more ornamented (Siefferman et al. 2005, Grindstaff et al. 2012), so is it not surprising that there are strong melanin ornament differences in the 2015 data compared to other years (Figure 10, Figure 11). This suggests that, even if there are differences between the sites, functionally within a site, ornamentation is likely indicating information on individual quality.

Additionally, for all collection years combined when limiting the data to similar dates as those surveyed in 2015, there was a significant correlation between date of plumage collection and plumage color, with birds caught earlier in the season having darker melanin plumage in both sexes at Camp Shelby only. As golf course birds received late, full, and early season plumage collection in 2012, 2013, and 2015 respectively, and Camp Shelby birds only received full and early season collection in 2014 and 2015, respectively, the lack of an individual late season examination of plumage ornaments in Camp Shelby birds may help explain these findings. Ornamentation may also fade throughout the season, and that is also a potential reason why it appears less ornamented birds
are caught later. It is also not unrealistic to hypothesize that this temporal variation seen in so-called ‘less ornamented’ birds would be observed later in the season, as bluebirds in other southern populations mating later in the season are in poorer condition (potentially unable to produce expensive ornaments) (Siefferman et al. 2005). It is unknown if this is the case for Camp Shelby birds given lack of data for late breeders in 2015.

**Male and Female Plumage Ornamentation**

Contrary to predictions, males and females express significantly higher quality ornaments (both UV and melanin) at the golf course compared to Camp Shelby for the entire study period. Past research on both melanin-based and structurally-based plumage coloration shows potential for condition dependence (McGraw et al. 2002) and both plumages are biologically produced via sensitive cellular molecular pathways (reviewed in McGraw, 2006) or nanostructural elements (Shawkey et al. 2003; Shawkey et al. 2006) that could respond to physiological disruption.

Insect quality or availability during molt may influence both plumages examined here (McGraw and Hill, 2006). There is the possibility that the golf course provides higher quality or more prey items for birds residing at the course throughout molt. As return rates are ~39% (2012-2013), it likely that a portion of the population undergoes molt on site. Unfortunately, I was unable to measure prey abundance at the golf course. Attempts to quantify insects via both sweep netting and pitfall traps were unsuccessful. Given ecologically relevant site
differences in open habitat and percent water (Chapter I), there is potential that sites differ in arthropod community diversity (reviewed in Begon et al. 2005). Additionally, previous research shows targeted elimination via pesticide treatment can also alter insect communities (reviewed in Newman, 2014). The quantity or quality of food offerings could be impactful here and should be assessed in the future.

Lastly, birds at the golf course may be supplementally fed as bluebirds are seen at bluebird feeders on residential properties adjacent to the golf course (L.M. Gillespie, pers. obs.). It is possible that human-provided food resources allowed the portion of returning golf course birds to produce energetically expensive ornaments in October (time of molt) of the preceding year. The majority of the birds on the golf course and Camp Shelby, however, are new unbanded birds each season and their previous environmental quality or nutrient access is unknown.

For newly assessed birds (ages and histories unknown) where they originated from can have significant impacts on ornamental plumage production and maintenance. For example, environment in the nest can influence bluebird color, as nestling males from smaller broods (that are fed more often) show brighter UV-blue coloration (Siefferman and Hill, 2007) while large brood environment increases T in both sexes of bluebird nestlings (Kozlowski and Ricklefs, 2011) potentially impacting ornaments. Developmental exposure to T and estrogen (E$_2$) may differentially impact normal sexual and social
development and influence adult reproductive function, courtship, aggressive, and territorial behaviors in songbirds (reviewed in Adkins-Regan, 2005; reviewed in Norris and Lopez, 2011). Reproductive measures, T and CORT are explored in depth in later chapters but there are plausible influences of these hormones on color differences stemming from the juvenile stage.

Male and Female UV Sexual Dimorphism: Between and within sites

The degree of color dimorphism was compared between sites, and for UV plumage (but not melanin), dichromatism is significantly lower for golf course compared to Camp Shelby birds (e.g. females express more ornamented plumage, Figure 13). Bluebird dichromatism is distinguishable at the nanostructural level, with males expressing more keratin rods similar in size, which yields higher UV chroma (Shawkey et al. 2003; Shawkey et al. 2006). This difference in dichromatism was clear in all years, not only in 2015. It may be that this result occurs because returning portions of golf course birds have access to supplemental food during molt. Meal worms are most commonly used in bluebird feeders, likely providing increases in caloric intake, not access to unique or higher quality nutrients (Doyle and Siefferman, 2014). However, not all birds return to breed, and overall, the golf course population is more ornamented, as such, on site nutrition access during molt cannot fully explain results for novel breeders at the course.

Only UV coloration is developed during the nestling period (not melanin-based plumage) and has been recently shown to be heritable in juvenile Florida
scrub-jays (*Aphelocoma coerulescens*) (Tringali et al. 2015). It is more likely that UV plumage differences within sites are due to genetic or influential developmental factors. Males are the default sex in birds and both sexes express different hormones in different target tissues at crucial periods of development (reviewed in Norris and Lopez, 2011) which could have impacts on coloration. Environmental factors (e.g. food stress, contaminants) can also influence steroid hormone production (Walsh et al. 2000; Saino et al. 2003; Honarmand et al. 2010) potentially compounding color differences. This explain the lower sexual dichromatism at the golf course, assuming that individuals were reared on this site, or that individuals lived for extended periods at this particular site, which potentially 9-39% of golf course birds do. Additionally, golf course females express higher levels of T (Chapter IV) and males lower T compared to Camp Shelby (Chapter III), and these hormone titers may also impact maintenance of coloration in general, and differentially within each sex.

**Conclusions**

Golf course males and females (for all study years combined) are significantly more ornamented; they express darker melanin breast color and greater UV tail chroma, contrary to specific predictions but consistent with the general hypothesis. Additionally, dimorphism in UV tail chroma differs significantly between sites; at the golf course, the sexes express more similar UV chroma than males and females at Camp Shelby.
These results are particularly interesting as sites are located 29.77 km apart and therefore experience the same yearly environmental (e.g. temperature, weather) variation. This indicates that plumage differences are potentially due to site-specific differences, and in this case, the golf course could be serving as an ecological trap (reviewed in Battin, 2004). Ecological traps are seemingly productive habitats, however, upon settling animals experience increases in juvenile mortality or difficulty locating high quality resources, for example (reviewed in Battin, 2004). Given bluebird preferences for nesting in open space habitats (Jones et al. 2014) and that the golf course provides this feature in abundance, this site may be attracting highly ornamented birds, however, after birds settle on site, they experience reduced productivity (reviewed in Battin, 2004; explored in Chapter’s IV and V). Populations here were not examined long enough for examination of this hypothesis, however, future research examining golf course habitats should utilize the ecological trap framework as it is vastly understudied, with meaningful application in the study of anthropogenic disturbance.
CHAPTER III – MALE BLUE RESPONSE TO CONSPECIFICS: DIFFERENCES IN HORMONES AND AGGRESSION ACROSS NESTING STAGES IN A POPULATION LIVING ON A SOUTHEASTERN GOLF COURSE

Introduction

Although birds live and breed in human-modified habitats, locations with greater human disturbance often have lower species richness (Beissinger and Osborne, 1982; Fernández-Juricic, 2000; Wood et al. 2015) and reproductive success of breeding birds in these habitats is typically lower (Tazelaar et al. 2013). Moreover, many species display behavioral adjustments in response to human presence, roads, traffic, human-made structures, and both noise and light pollution (Barber et al. 2010; reviewed in Sol et al. 2013). Urbanization continues to increase, resulting in increased environmental alteration of previously undisturbed habitat. Reports of maladaptive impacts of human disturbance on behavior (reviewed in Clotfelter et al. 2004; Carere et al. 2010) and physiology (reviewed in Frye et al. 2012; reviewed in Shenoy and Crowley, 2011) are becoming more common in the literature.

Stress and Reproductive Hormones

In avian studies, behavioral and physiological responses to disturbance often focus on stress hormones (glucocorticoids or GCs) via examination of either baseline or stress induced increased corticosterone (CORT, main avian GC) levels. As discussed in Chapter 1, in addition to responding to stress, CORT also facilitates daily functions to access energy stores, and as such is always
present in the blood (termed predictive homeostasis or baseline levels, (Romero, 2004)). Baseline levels of GCs vary daily and annually as energetic demands fluctuate, however, they are also used in ‘stress’ responses and can become elevated over short periods. Where an organism’s homeostatic state is disrupted, the animal undergoes a CORT-mediated stress response and hormone levels in this situation can increase drastically (termed reactive homeostasis, stress-induced levels, (Romero, 2004)). If the stressor is prolonged, the response becomes maladaptive and can affect reproduction, e.g. producing fewer young (Adkins-Regan, 2005; Nelson, 2011; Ouyang et al. 2015).

An individual’s physiological condition may influence CORT responses to disturbance (Beale and Monahan, 2004), and birds responding to disturbance with greater increases in CORT tend to have passive behavioral responses that are thought to allow them to cope better with environmental change (Cockrem, 2013). The risk-disturbance hypothesis predicts that physiological responses of disturbed animals (e.g. CORT responses) will be greater with increased perceived predation risk and stage of reproductive investment (reviewed in Frid and Dill, 2002). Predictions based on the risk-disturbance hypothesis can be applied to both between and within sites, as birds in more disturbed locations would be expected to respond to disturbance events with increased aggression, CORT, and possibly testosterone (reviewed in Frid and Dill, 2002). Within sites, the hypothesis also predicts that aggression (and possibly hormones) may
increase across the nesting cycle, paralleling increasing reproductive investment (reviewed in Frid and Dill, 2002).

Studies of avian GC responses demonstrate that urban environments can select for flexible stress responses in populations (Partecke et al. 2006), although the strength and direction of relationships between CORT and urbanization are equivocal and may be complicated by variation due to sex and life history stage. Researchers investigating GCs and disturbance do not always investigate other hormones, and studies also investigating androgens along with CORT are less common. Androgens, (e.g. testosterone, T) do not always show the same daily or seasonal patterns as CORT (reviewed in Bonier, 2012) but they too fluctuate. While both help to regulate physiology and behaviors in stressful interactions, T also regulates behaviors and physiology associated with reproduction.

Reproductive Behaviors and T. Aggression enables individuals to compete for and defend limited breeding territories, resources, and mates. It has been well documented that males with higher levels of T have increased reproductive success (reviewed in Adkins-Regan, 2005; reviewed in Møller et al. 2005). The typical T profile for male songbirds tends to be elevated as the breeding season commences, when aggressive and courtship interactions occur, and then drops during parental care stage (Beecher et al. 1997; Beletsky et al. 1990; Jawor, 2007; Ketterson et al. 2009). The Challenge Hypothesis (Wingfield et al. 1990) postulates that T in males may acutely elevate in the presence of same sex conspecifics, facilitating aggressive interactions when it may more directly impact
fitness. This hypothesis is often tested in laboratory and field experiments using simulated territorial intrusions (STIs). Researchers present focal birds with decoy or live, caged ‘intruders’ and assess both behavioral response and hormone levels. To date, males of many bird species respond to these staged encounters with increased circulating T although for others this relationship is more complex and the hypothesis is still debated by some (reviewed in Goymann et al. 2007).

Studies using implants of exogenous T demonstrate how T can influence male territorial, breeding, and parental care behaviors. Males implanted with T often have larger home ranges and defend larger territories (Wingfield, 1984; Chandler et al. 1994), and generally show increased aggression, male-male competitive ability or dominance (Hegner and Wingfield, 1987; Collis and Borgia, 1992). Males with T implants tend to be more attractive to females (Enstrom et al. 1997) and gain higher reproductive output through increased polygyny or extra pair fertilizations (Wingfield, 1984; Raouf et al. 1997). Exogenous T can lead to poor defense of young against nest predators and reduced nestling provisioning (Hegner and Wingfield, 1987; Chandler et al. 1997; Cawthorn et al. 1998; Clotfelter et al. 2007). Sometimes, there are no impacts of experimentally elevated T on behavior or conspecific aggression (Chandler et al. 1994; Apfelbeck and Goymann, 2011) suggesting a complex association between T and behavior. At a proximate level, T implants have been found to increase activation of high numbers of sexually dimorphic genes in the hypothalamus and
amygdala (Peterson et al. 2013) suggesting a mechanism for the individual variation seen in the T-behavior association.

**Endocrine and Behavioral Profiles and Disturbance**

Both T and CORT can co-vary (reviewed in Adkins-Regan, 2005) and both may influence aggressive behavior, however, the examination of associations between these traits in relation to human-modified environments are uncommon. Recently, researchers have shown that behavior and endocrine profiles differ between birds living in urban and rural habitats, although the strength and direction of relationships vary (reviewed in Sol et al. 2011; reviewed in Bonier, 2012). Urban-living song sparrows (Scales et al. 2011) display increased boldness and aggression compared to birds in rural areas. Moreover, rural populations demonstrate strong correlation between boldness and aggression while urban populations do not (Scales et al. 2011). Behavior may not be similarly expressed in urban vs. rural populations suggesting population level adaptations of behavioral types and that selection associated with urbanization can disrupt behavioral phenotypes (Bókony et al. 2012).

Eastern bluebirds (*Sialia sialis*) were preferentially introduced to human-modified breeding locations following reduction of open, grassy, semi-wooded areas that nearly led to their demise in the middle of the 20th century (Gowaty and Plissner 1998). Nest boxes provided by backyard-birders restored population numbers, and many of these ‘box trails’ were placed in golf courses, cemeteries, and recreational parks (Gowaty and Plissner 1998). These areas can be heavily
impacted by human presence and activity, habitat modification, lawn maintenance activity and application of pesticides, all of which could influence physiology and behavior.

*Eastern Bluebirds*

Bluebirds are sexually dimorphic with males exhibiting bright, ultra-violet (UV) blue structurally-based coloration on the head, back, wings and tail and a reddish-brown melanin-based plumage patch on the breast. Males compete aggressively for access to nest boxes and mates, and those with the most ornamented UV-blue coloration are more successful in acquiring nest resources (Siefferman and Hill, 2005b). More-ornamented males (both brighter UV blue plumage and darker breast patches) pair with females that breed earlier in the season, provision young more often, and fledge larger young (Siefferman et al. 2005; Grindstaff et al. 2012). Ultra-violet plumage likely signals aggression as males are more aggressive toward decoys that have more colorful UV plumage (greater UV chroma) (Mercadente and Hill, 2014). Both plumages are condition dependent (for full detail, see Chapter II) and in an Alabama population, males that display less ornamented breast color exhibit higher T (Siefferman et al. 2013). In an Oklahoma population, males with brighter UV coloration have both higher CORT and lower T (Grindstaff et al. 2012).

The extent to which human disturbance influences reproductive output in eastern bluebirds is less clear. Anthropogenic disturbance in bluebirds can results in delayed breeding (Stanback and Siefert, 2005) or altered productivity
In a VA bluebird population, increased anthropogenic noise results in decreased self-maintenance behaviors and altered song (Kight and Swaddle, 2007; Kight and Swaddle, 2012; Kight and Swaddle, 2015). Experimentally increased human disturbance caused increased pair aggression toward a non-competitive hetero-specific in western bluebirds (*Sialia mexicana*) (Bhardwaj et al. 2015).

Nest box density or habitat features can also impact bluebird behavior, specifically aggression, with birds nesting in areas of higher box density displaying greater aggression (Bhardwaj et al. 2015). The amount of open habitat within the territory can predict bluebird site occupancy; bluebirds seem to prefer more open habitat, particularly when breeding in environments with increased interspecific competition (Jones et al. 2014).

Here, I investigate associations between conspecific aggression, T, CORT, and offspring production of males between two sites, a golf course and a rural military base (sites fully reviewed in Chapter I). These sites differ in levels of human disturbance and habitat characteristics; the golf course has significantly higher human traffic, percent open habitat, percent water, and nest box density than Camp Shelby (Chapter I). Additionally, golf course males display more highly ornamented plumage (Chapter II). I use simulated territorial intrusions during different life history stages (nest building and incubation) to assess aggression. I hypothesize that increased disturbance will subtly effect the
relationships between hormones, behavior and ornamentation in eastern bluebirds.

Predictions between Sites

In keeping with the risk-disturbance hypothesis and because there are site differences in both open habitat, next box density, and land management practices combined with chronic and unpredictable human activity (Chapter I) I predict that males at the golf course population will 1) Respond to intrusions with increased aggression due to nest boxes being more densely situated at the golf course (Bhardwaj et al. 2015), 2) Display lower levels of T (reviewed in Shenoy and Crowley, 2011) and higher levels of CORT (reviewed in Frid and Dill, 2002) due to stresses associated with nest defense and the interactions between CORT and T noted in Adkins-Regan (2005), 3) Experience reduced reproductive output due to variation in stress physiology, nesting resources, increased human disturbance and 4) Show different relationships among hormones, behavior, plumage and reproductive indices within sites compared to males breeding in the more rural and natural habitat.

Predictions within Sites

Within sites, more ornamented individuals (e.g. higher UV tail chroma and darker melanin breast pigment) are predicted to 1) Display higher CORT and lower T as in Grindstaff et al. (2012), 2) Respond to intrusions with increased aggression (Mercadente and Hill, 2014), and 3) Fledge more offspring (Siefferman et al. 2005). Under the risk disturbance hypothesis (reviewed in Frid
and Dill, 2002), individuals living in more disturbed locations, such as the golf course, will experience increases in 4) Response T, 5) Response CORT, and 6) Response aggression across the nesting cycle (from nest building to incubation) (‘Response T/CORT’ designates results or discussion in reference to results across nesting within sites as opposed to comparisons between them; the measure itself, hormone response to STI, is the same). Under the challenge hypothesis (Wingfield et al. 1990), males are expected to respond to conspecific intrusions with higher response T during nest building compared to incubation.

Methods

Study Sites

High Disturbance Location. The full extent of the differences between the study sites is described in Chapter I. Briefly, the high human disturbance population of bluebirds breed at a golf course (Hattiesburg, MS; 31 20.9’ N, 89 22.6’ W). There is significantly more open habitat (open space with short grass; preferable to bluebirds) and water at this site, however, the turf is maintained daily, kept short, and pesticides are applied (Chapter I, Table 1). Nest boxes here experience significantly higher human foot traffic and are more densely located (Chapter I). I monitored 45 boxes at this site.

Low Disturbance Location. The low disturbance site is rurally located Camp Shelby Joint Forces Training Center (Camp Shelby, Hattiesburg, MS). There is only annual mowing of grounds, human presence and foot traffic is rare, and few nest boxes examined and included for study experience significantly
high vehicle traffic (Chapter I). This site has more roads and buildings and boxes are less densely arranged. Prior to the study, 250 bluebird boxes were erected on the military base; I monitored 120 nest boxes and avoided using those boxes in high traffic areas.

*Bird Capture and Monitoring*

Bluebirds at the field sites begin prospecting for territories and mates in February and March and breed until August. Bluebirds produce up 2-3 successful broods in southern populations (average species clutch size 4-5 eggs). I captured bluebirds from 0400-1200h using mist nets or box traps. I measured mass, tarsus length, wing chord, tail length and banded birds with a USFWS identification band (permit #23479-C) and a unique combination of color bands. In 2013, I followed birds at the golf course while, in 2014, I followed birds at Camp Shelby. In 2015, I monitored both field sites simultaneously. I monitored nest boxes daily to document nest building and monitored nests every other day for initiation of egg laying. Once eggs were laid and/or nestlings hatched, I monitored nests every 3 days.

*Behavioral Assays*

I performed two, same sex, simulated territorial intrusions (STIs) using male conspecifics during the nest building period (NBI) and during the incubation period (II; the second week of incubation). During these intrusions, I placed a male (caught elsewhere on site) in a small wire cage 0.3m from a focal pair’s nest-box. I broadcasted eastern bluebird song and chatter. For 10 min, I
recorded the following information: latency time until 1) each adult arrived (within 40m of the nest box); 2) the time of first attack, and the total number of times the male A) attacked, B) dove, C) flew to the nest box, and D) landed on top of the intruders cage. After the 10 min STI, I attempted to capture the male and female (females discussed in Chapter 4). I continued the broadcast of bluebird vocalizations up to 30 min after the STI. Upon capture, I took blood from the brachial vein within 3 min of handling. I stored blood in a cooler prior to being centrifuged, plasma was extracted and stored it at -20°C.

*Hormone Assays, Testosterone*

I measured T concentrations using enzyme linked immunosorbent assays (ELIZA; Enzo Life Sciences) following methods outlined in Jawor (2007). First, I extracted hormones using diethyl-ether (3x), and then resuspended, and diluted extracts with ethanol (50µl) and assay buffer (300µl). I combined plasma samples (30µl) with tritiated testosterone (2000 cpm, H\(^3\)T, PerkinElmer) to allow for calculation of hormone recovery percentages (mean recoveries = 72%). I calculated concentrations of T and corrected for incomplete recoveries (Microplate Manager, Bio Rad Laboratories; H1 Synergy Reader, BioTek). I analyzed multiple samples from a single individual on the same plate, and randomly located sample placement on plates. I calculated intra-assay and inter-assay variation by randomly placing standards throughout the plate; intra-assay variation was 1-24% while inter-assay variation was 6%.
Hormone Assays, Corticosterone

I conducted CORT analyses using an enzyme linked immunosorbent assay (Arbor Assays, LLC) as outlined in DeVries and Jawor (2013). As with T, I extracted CORT using diethyl-ether (3x), and resuspended extracts with assay buffer. I combined plasma samples (10ul) with tritiated CORT (2000 cpm, \( \text{H}^3 \text{CORT}, \text{PerkinElmer} \)) to allow for calculation of hormone recovery percentages (mean recoveries = 76%). I calculated concentrations of CORT and corrected for incomplete recoveries (Microplate Manager, Bio Rad Laboratories; H1 Synergy Reader, Bio Tek Laboratories). Multiple plasma samples for single individuals were analyzed on the same plate, and sample placement was random throughout plates. Intra-assay and inter-assay variation were calculated by randomly placing standards throughout the plate; northern bobwhite quail (\textit{Colinus virginianus}) homogenized plasma served as a standard for this assay; intra-assay variation was 1-17% while inter-assay variation was 24%. Inter-assay variation inflation is due to the use of multiple plates from multiple kits.

Plumage Analysis

Spectrometry was used to measure light reflectance from plumage samples (reviewed in Andersson and Prager, 2006). Spectrometers used were tuned to the specific wavelengths (see Chapter II for full detail) for UV and melanin pigment analysis, respectively, as this method results in increased accuracy of reflectance measures, specifically for melanin pigments (reviewed in Andersson and Prager, 2006).
UV Tail Chroma. Samples were sent to Dr. Lynn Siefferman (Appalachian State) for analysis using a S2000 spectrometer (range 250–880 nm; Ocean Optics, Dunedin, FL). Replicate measurements from the same area of each tail feather sample and were collected and subsequently averaged. Ultra-violet chroma (spectral purity) was calculated (methods outlined in detail in Chapter II).

Melanin Pigment. A S2000 spectrometer (range 300-700nm; Ocean Optics, Dunedin, FL) was also used to take measurements from each breast feather sample. Mean brightness (amount of light reflected from plumage sample; for full detail see Chapter II) was calculated for melanin pigment (methods outlined in detail in Chapter II).

Statistics

I used SPSS (version 23.0) for data analysis. Most data tested for normalcy using Shapiro-Wilk were not normally distributed and I either log transformed or standardized (using z-scores) for year for use of parametric tests. Both independent and paired samples t-tests were used to examine variables between sites and between life history stages, respectively. Significant results from previous analyses justify correlating plumage interactions between variables separately within sites using Spearman’s Correlations (Chapter II). Results presented are for site variation in STI elicited T, CORT, and aggression, differences in offspring fledged, and within site correlations of these variables to both melanin and UV plumages. To control for false discovery rates as multiple comparisons were used to correlate traits within sites, a stepwise, Benjamini-
Hochberg procedure (Benjamini and Hochberg, 1995) was used to sequentially reduce the number of comparisons, and the false discovery rate was set to 10% (McDonald, 2014).

Results

Handling and Capture

For this analysis all types of intrusion were combined to determine if handling (removing birds from the mist net and processing of blood sampling) or time it took to capture individuals (e.g. capture time; the time elapsed from 10 min observation session to when bird was captured in net) influenced hormone levels. On average, all birds were bled in within 3 min of capture (mean=2.13, N=122, SD=0.936). Spearman correlations within Camp Shelby and the golf course reveal there was no effect of handling time on T or CORT (Table 9). At the golf course, there was no effect of capture time on T or CORT (Table 9). At Camp Shelby, there was no correlation between T and capture time but there was a significant correlation between CORT and capture time (Table 9). To standardize CORT by capture time, a linear regression was performed and the standardized residual was used in analyses.

Table 9

Handling and Capture Effects on Hormones

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Golf Course</th>
<th></th>
<th></th>
<th>Camp Shelby</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
<td>N</td>
<td>rs</td>
<td>p</td>
<td>N</td>
</tr>
<tr>
<td>T*Bleed Time</td>
<td>-0.002</td>
<td>0.990</td>
<td>44</td>
<td>-0.140</td>
<td>0.283</td>
<td>60</td>
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<tr>
<td>CORT*Bleed Time</td>
<td>-0.117</td>
<td>0.467</td>
<td>41</td>
<td>-0.017</td>
<td>0.894</td>
<td>62</td>
</tr>
<tr>
<td>T*Capture Time</td>
<td>-0.227</td>
<td>0.116</td>
<td>49</td>
<td>-0.016</td>
<td>0.901</td>
<td>60</td>
</tr>
</tbody>
</table>
Table 9 (continued).

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td>CORT* Capture Time</td>
<td>rs p N</td>
<td>rs p N</td>
</tr>
<tr>
<td></td>
<td>-0.099 0.495 50</td>
<td>0.449 &lt;0.0001 61</td>
</tr>
</tbody>
</table>

Raw data is used in this analysis; rs = Spearman’s rho, correlation coefficient; N= sample size; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons. Bleed Time = time elapsed from net removal to cessation of blood sampling; Capture Time = time elapsed from cessation of behavioral observation to bird’s capture in mist net.

**Annual Variation in Reproductive Indices, Hormones, and Behavior**

For offspring fledged, there were no significant differences between years at the golf course or at Camp Shelby (Table 10). For birds captured during simulated territory intrusions (both types combined) there were no significant differences between years for T, CORT, or attacks at the golf course or Camp Shelby.

Table 10

**Annual Variation within Sites**

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Year</td>
<td>N</td>
</tr>
<tr>
<td>Offspring Fledged</td>
<td>2012</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>24</td>
</tr>
<tr>
<td>T</td>
<td>2013</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>13</td>
</tr>
<tr>
<td>CORT</td>
<td>2013</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>25</td>
</tr>
</tbody>
</table>

N=sample size; F(df) = test statistic for ANOVA; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparison.
Site Comparisons of Offspring Fledged

There were no differences in number of offspring fledged between the golf course (M=0.47, SD=0.18, N=70) and Camp Shelby (M=0.52, SD=0.20, N=42) ($t=-1.14$, DF=110, $p=0.26$) (Figure 14).

Site Comparisons of T, CORT, and Aggression

Golf course males display significantly lower T concentrations than Camp Shelby males during nest building but not during incubation intrusions (Table 11, Figure 15). During both nest building and incubation intrusions, CORT responses did not differ significantly between golf course and Camp Shelby males (Table 11, Figure 16). Golf course males displayed significantly less aggression than Camp Shelby males during nest building intrusions. During incubation, aggression did not differ significantly between golf course and Camp Shelby males (Table 11, Figure 17).
Figure 14. Offspring Fledged: All Years

Number of Offspring= offspring fledged from first nest of the season. Raw data is used in graph. The golf course is represented by the white bar and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and the open circles are beyond the 10th and 90th percentiles.

Table 11

Site Comparisons: Hormones, Behavior, and Lift History Stage

<table>
<thead>
<tr>
<th>Nest Building</th>
<th>Golf Course</th>
<th></th>
<th>Camp Shelby</th>
<th></th>
<th>Independent samples t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>n</td>
<td>M</td>
<td>SD</td>
</tr>
<tr>
<td>T</td>
<td>-0.14</td>
<td>0.41</td>
<td>34</td>
<td>0.23</td>
<td>0.30</td>
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<tr>
<td>CORT</td>
<td>1.55</td>
<td>0.28</td>
<td>33</td>
<td>1.62</td>
<td>0.21</td>
</tr>
<tr>
<td>Aggression</td>
<td>0.77</td>
<td>0.81</td>
<td>39</td>
<td>1.23</td>
<td>0.85</td>
</tr>
<tr>
<td>Incubation</td>
<td>M</td>
<td>SD</td>
<td>n</td>
<td>M</td>
<td>SD</td>
</tr>
<tr>
<td>T</td>
<td>-0.01</td>
<td>-0.55</td>
<td>15</td>
<td>-0.18</td>
<td>0.34</td>
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<tr>
<td>CORT</td>
<td>1.51</td>
<td>0.22</td>
<td>18</td>
<td>1.48</td>
<td>0.16</td>
</tr>
<tr>
<td>Aggression</td>
<td>0.81</td>
<td>0.81</td>
<td>28</td>
<td>1.26</td>
<td>0.84</td>
</tr>
</tbody>
</table>

M=mean; SD= standard deviation; n=sample size; t = test statistic; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparison.
Figure 15. T Response to Intrusions between Sites

Raw data is used in graph. The golf course is represented by the white bar and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and the open circles are beyond the 10th and 90th percentiles.
Figure 16. CORT Response to Intrusions between Sites

Raw data is used in graph. The golf course is represented by the white bar and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and the open circles are beyond the 10th and 90th percentiles.

Conspecific Intrusions: Variation between Life History Stages within Sites

There were significant increases in response T concentrations to STI from nest building to incubation within the golf course (Figure 15, Table 12). Within Camp Shelby, males showed decreased circulating response T concentrations to STI from nest building to incubation (Figure 15, Table 12). There were significant decreases in circulating response CORT concentrations to STI from nest building to incubation at the golf course but not at Camp Shelby (Figure 16, Table 12).
There were significant increases in response aggression displayed between intrusion type at the golf course and Camp Shelby (Figure 17, Table 12).

![Graph showing aggressive response to intrusions between sites]

**Figure 17. Aggressive Response to Intrusions between Sites**

Attacks toward intruder = total attack number within 10 min observation period. Raw data is used in graph. The golf course is represented by the white bar and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and the open circles are beyond the 10th and 90th percentiles.

**Table 12**

<table>
<thead>
<tr>
<th>Nest Building and Incubation within Sites: T, CORT, Aggression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paired Samples t-test</strong></td>
</tr>
<tr>
<td><strong>Golf Course</strong></td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>T</td>
</tr>
<tr>
<td>CORT</td>
</tr>
<tr>
<td>Aggression</td>
</tr>
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</table>
Table 12 (continued).

<table>
<thead>
<tr>
<th></th>
<th>M</th>
<th>SD</th>
<th>N</th>
<th>T</th>
<th>df</th>
<th>P</th>
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<tbody>
<tr>
<td><strong>Camp Shelby</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>1.40</td>
<td>0.77</td>
<td>61</td>
<td>14.12</td>
<td>60</td>
<td>&lt;0.0001*</td>
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<tr>
<td>CORT</td>
<td>-0.13</td>
<td>0.60</td>
<td>62</td>
<td>-1.67</td>
<td>61</td>
<td>0.101</td>
</tr>
<tr>
<td>Aggression</td>
<td>0.21</td>
<td>0.97</td>
<td>75</td>
<td>1.84</td>
<td>74</td>
<td>0.07*</td>
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</tbody>
</table>

M=mean; SD=standard deviation; n=sample size; means and standard deviations presented in this table are for log transformed data; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.

**Relationships among Variables within Sites**

Spearman correlations within the golf course show that males nesting in areas with a higher percentage of open habitat fledged significantly fewer nestlings (Table 13). Golf course males displaying higher quality (e.g. darker) melanin ornaments fledge more offspring (Figure 18, Table 5), however, golf course males displaying lower quality UV ornaments fledge more offspring (Figure 19, Table 13). There are no relationships between ornamentation and open habitat, density, T, CORT, or aggression within Camp Shelby (Table 13).
Figure 18. Melanin Ornament and Offspring Fledged at the Golf Course

Raw data is used in graph. For melanin ornaments, a lower number indicates a darker bird, which is a more ornamented bird.
Figure 19. UV Ornament and Offspring Fledged at the Golf Course

Raw data is used in graph. For UV chroma, a higher number indicates a higher quality ornament (e.g. more saturated).

Table 13

Relationships to Ornamentation within Sites

<table>
<thead>
<tr>
<th>Habitat</th>
<th>Golf Course</th>
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<th></th>
<th>Camp Shelby</th>
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<tr>
<td></td>
<td>rs</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habitat*Offspring</td>
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<td>0.003*</td>
<td>54</td>
<td>-0.169</td>
<td>0.302</td>
<td>39</td>
</tr>
<tr>
<td>Fledged</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Open Habitat*T</td>
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<td>0.272</td>
<td>45</td>
<td>0.053</td>
<td>0.684</td>
<td>61</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Habitat*CORT</td>
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<td>0.02</td>
<td>45</td>
<td>-0.189</td>
<td>0.160</td>
<td>57</td>
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<td></td>
</tr>
<tr>
<td>Habitat*Attack</td>
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<td>0.345</td>
<td>67</td>
<td>-0.066</td>
<td>0.577</td>
<td>75</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
<td>N</td>
<td>rs</td>
<td>p</td>
<td>N</td>
</tr>
<tr>
<td>Density*CORT</td>
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<td>0.05</td>
<td>43</td>
<td>-0.048</td>
<td>0.710</td>
<td>62</td>
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<tr>
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<td>43</td>
<td>-0.059</td>
<td>0.667</td>
<td>56</td>
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<tr>
<td>Density*Attack</td>
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<td>65</td>
<td>0.036</td>
<td>0.766</td>
<td>70</td>
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Table 13 (continued).

<table>
<thead>
<tr>
<th></th>
<th>Golf Course</th>
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<tr>
<td></td>
<td>$r_s$</td>
<td>$p$</td>
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<tr>
<td><strong>Density</strong></td>
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<tr>
<td><em>Offspring Fledged</em></td>
<td>0.311</td>
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<tr>
<td><strong>T</strong></td>
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<td></td>
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<tr>
<td><em>Offspring Fledged</em></td>
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<td>0.230</td>
</tr>
<tr>
<td><em>Attack</em></td>
<td>0.188</td>
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<tr>
<td><em>UV</em></td>
<td>-0.093</td>
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<tr>
<td><em>Melanin</em></td>
<td>-0.037</td>
<td>0.812</td>
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<td>0.117</td>
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<td><em>Offspring Fledged</em></td>
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<tr>
<td><em>Attack</em></td>
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<td><em>UV</em></td>
<td>0.100</td>
<td>0.500</td>
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<td>0.742</td>
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<td><strong>Offspring</strong></td>
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<tr>
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<tr>
<td><em>Fledged</em> UV</td>
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<td>0.011*</td>
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<tr>
<td><em>Fledged</em> Melanin</td>
<td>-0.35</td>
<td>0.013*</td>
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<tr>
<td><strong>Ornaments</strong></td>
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<tr>
<td><em>UV</em> Attack</td>
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</tr>
<tr>
<td><em>Melanin</em> Attack</td>
<td>0.079</td>
<td>0.563</td>
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</table>

Raw data is used in this analysis; $r_s$ = Spearman’s rho, correlation coefficient; $N$= sample size; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.

Discussion

Results here demonstrate support for the hypothesis that differences in level and type of anthropogenic disturbance subtly alter physiology and behavior in male bluebirds. Golf course males had significantly lower T during nest building, but not incubation intrusions, when compared to Camp Shelby males.
Golf course males also responded with lower levels of aggression during nest building, but not incubation intrusions, compared to Camp Shelby males. However, males showed no covariation between T and aggression within sites. Within the golf course, males with *more ornamented* melanin pigmented breasts, and those with *less ornamented* UV tail chroma, fledged more offspring and these relationships were not present at Camp Shelby.

Predictions based on the risk-disturbance hypothesis for high CORT responses (reviewed in Frid and Dill, 2002) were not supported in the golf course population, and while there were differences in CORT across the nesting cycle, they were not as expected as both populations displayed decreased response CORT across the nesting cycle, contrary to predictions (reviewed in Frid and Dill, 2002). Golf course males also displayed decreased response aggression across the nesting cycle, contrary to predictions of aggressive responses paralleling increasing reproductive investment (reviewed in Frid and Dill, 2002), as was the case in Camp Shelby males. These results are interesting given the significant differences between sites in human activity and small vehicle traffic (Chapter I); this suggests bluebirds show behavioral flexibility in aggressive responses, potentially responding differently to human vs. vehicle disturbance elements within a human modified environment.

**Conspecific Intrusions**

*Corticosterone.* There were no significant differences in CORT between sites during nest building or incubation intrusions, however, golf course males
demonstrated CORT levels within a larger range than Camp Shelby males (Figure 16). Males at the golf course were predicted to display higher CORT (reviewed in Frid and Dill, 2002) response to STI in comparison to Camp Shelby, the less disturbed population (reviewed in Frid and Dill, 2002). Research on patterns of both baseline and stress induced CORT in human-disturbed populations, however, are inconsistent regarding the sensitivity and direction of responses (reviewed in Bonier, 2012) as CORT increases or decreases based on species examined.

While golf course males responded with a larger range of CORT levels overall, at the golf course only, males showed decreased CORT levels from nest building to incubation intrusions, contrary to predictions consistent with the risk disturbance hypothesis. These results are interesting as CORT could be predicted to increase at this time to prepare for nestling provisioning (Bonier et al. 2009; Bonier et al. 2011). One of the main, non-stress related functions of CORT is to allow access to energy stores via glucose release (Nelson, 2011), something that actively breeding birds might need more access to. It is also possible that the incubation stage is not particularly energetically expensive for males and if CORT had been measured during the nestling rearing stage, increases in CORT may have been detected.

Testosterone. Testosterone levels measured in response to conspecific intrusions reveal golf course males respond with significantly lower T than males at Camp Shelby during nest building but not incubation (Figure 15). Whether
simulated territorial intrusions trigger elevated T from baseline appears to vary with species and may depend on mating systems or nesting ecology (Wingfield et al. 1990). Although I did not measure baseline versus elevated T in the same males, preliminary data measured during nestling feeding suggest that males captured passively have lower mean T compared to males that were bled after experiencing simulated intrusions (unpublished data, L.M. Gillespie). Those results should be interpreted carefully, however, given that the challenge hypothesis also predicts decreases in T during nestling feeding (Wingfield et al. 1990; Jawor, 2007).

In response to STI, males of some species elevate T (Wingfield and Wada, 1989; Gwinner et al. 2002) and males of other species do not (Fedy and Stutchberry, 2006; DeVries et al. 2012; Fokidis et al. 2011; Villavicencio et al. 2014). Interspecific variation between T elevation and aggression is an ongoing topic of research interest (Goymann et al. 2007; Goymann and Wingfield, 2014). Male bluebirds responding to a hetero-specific (house sparrow) show lower overall androgens, however, blood samples were not collected directly after behavior nor correlated with it, and likely better reflect male ornamentation (Grindstaff et al. 2012).

The lower T response of golf course males compared to Camp Shelby males was partially supported as predicted based on previous findings (reviewed in Shenoy and Crowley, 2011) for nest building, but not for incubation (Figure 15). That golf course males increase response T across breeding stages, from
nest building to incubation, is consistent with predictions for the risk disturbance hypothesis (reviewed in Frid and Dill, 2002). This hypothesis predicts increased investment across nesting will result in more intensive responses to disturbance events in disturbed populations, specifically predicting higher CORT paralleling increasing investment (reviewed in Frid and Dill, 2002). Based on this prediction of higher CORT, higher T across nesting may be predicted as a territorial intrusion is both a stressful and aggressive situation, and T and CORT exert similar physiological impacts advantageous in situations of this nature (reviewed in Adkins-Regan, 2005).

Previous research has shown that circulating levels of hormones may not determine behavior, as either androgen receptor number or distribution in the brain can vary (reviewed in Adkins-Regan, 2005; Nelson, 2011). It may be that golf course males develop increases in receptor number or distribution, and thus may not require high levels of T to facilitate aggressive interactions. This may be adaptive when disturbance events are chronic and unpredictable (Chapter I), as substantially increasing T upon every conspecific intrusion (or disturbance event) could result in maladaptive impacts of high T. Thus, higher human activity in this environment (Chapter I) may help select for decreased T responsiveness as an adaptive mechanism. Alternatively, it is possible that differences in land maintenance practices, which are an issue at many locations like golf courses, could potentially explain these relationships and remain a viable topic for future research.
Previous research on breeding season hormonal profiles of male birds shows that many species display reduced T during phases where paternal care is required (Wingfield et al. 1990; Ketterson et al. 2009). Incubation intrusions were performed within 7 days prior to hatch, and Camp Shelby male’s decrease in response T from nest building to incubation is likely adaptive. Nest survival requires male attendance and provisioning of young, which in other species, is negatively impacted by exposure to elevated levels of T (reviewed in Adkins-Regan, 2005). That golf course males increase T from nest building to incubation is interesting, and could be due to increases in conspecific density as breeding progresses, resulting in increased conspecific interactions. Western bluebirds, a close relative, adjust aggression in relation to competition for nest boxes with other species (Bhardwaj et al. 2015), and higher T may help facilitate defense (especially of high quality territory) as nest box saturation occurs at the golf course.

Based on the challenge hypothesis, T levels were predicted to be higher during nest building than incubation (Wingfield et al. 1990), however, this hypothesis is only supported by results among Camp Shelby males displaying significantly higher response T during nest building, but not incubation (Figure 15). Camp Shelby males are hypothesized to reside in the more ‘natural’ habitat, as such, their within-site T decrease between nest building and incubation is likely adaptive. Bluebirds are often double-brooded at both sites, but sometimes triple-brooded at Camp Shelby only. As such, Camp Shelby males may need
tighter endocrine regulation of parental care (e.g. strict decreases in T at certain times) in order to be capable of having enough energy to invest and to facilitate increased parental care behavior over a prolonged breeding season.

Differences exist between sites in T and they do so in unique patterns. While golf course males increase T from nest building, levels are still significantly lower than Camp Shelby levels, and that it does not correlate with increases in aggression are all consistent with the hypotheses that behavior is insensitive to T and regulated through some other mechanism. Perhaps this is the case for all bluebirds, or perhaps golf course bluebirds have adapted these physiological mechanisms in response to their environment. Hormone manipulation or examination of receptor distributions needs to occur to answer these questions in bluebirds.

Aggression. Males at the golf course display significantly lower levels of aggression towards conspecifics compared to Camp Shelby males (Figure 17). These results are contrary to predictions of the risk disturbance hypothesis (reviewed in Frid and Dill, 2002). This hypothesis predicts increased aggressive responses to disturbance events, however, it hinges upon an animal’s perceived predation risk (e.g. continually perceiving humans as predators while experiencing chronic human interaction can be maladaptive) (reviewed in Frid and Dill, 2002). These results are also inconsistent with research demonstrating that, in many songbird species, urban living birds display more aggressive,
territorial behavior and are more bold (Fokidis et al. 2011; Scales et al. 2011; reviewed in Bonier, 2012).

Lastly, aggression in bluebird populations seems to vary with geographic location (Lynn Siefferman, pers. comm.), indicating differences in aggression may be both adaptive and flexible in this species. Given this variability, results of this study suggest that even populations that are close geographically (see Chapter I, Figure 1) can display extreme differences in physiology, behavior, and ornamentation that may be a consequence of differing exposures to both human activity and modified habitat (Chapter I).

Relationships within sites to Ornamentation

Within the golf course only, males nesting in areas with a higher percentage of open habitat fledged significantly fewer nestlings. This is surprising given bluebird historic preference for open, short grass habitat (Gowaty and Plissner, 1998; Jones et al. 2014). At the golf course, however, open habitat equates to boxes located close to the green, which are areas experiencing increased human activity at this site (Chapter I; pers. obsv. LM. Gillespie). So while this may, at first, appear as high quality habitat, potentially birds experience increased stress living here and thus experience lower reproductive output. These trends may be explained by ecological traps (Gates and Gysel, 1978), in which animals make poor choices in habitat settlement due to environmental cues that were once associated with high quality but have now been altered by human disturbance (Schlaepfer et al. 2002). The missing environmental
requirements negatively impact reproduction and potentially adult physiology and behavior.

Also only within the golf course males displaying higher quality melanin ornaments (darker, more pigmented) fledge more offspring (Figure 18). However, golf course males displaying lower quality UV tail chroma also fledge more offspring (Figure 19). Results for melanin ornaments correlating with increased reproductive success within golf course males are consistent with other research in another southern population for melanin ornamentation (Siefferman et al. 2005), but surprisingly, not for UV ornamentation. Other bluebird populations show correlations of brighter and more chromatic UV ornaments to measures of increased reproductive success (Siefferman et al. 2005; Grindstaff et al. 2012), while golf course males displaying lower UV tail chroma (e.g. less ornamented) experienced increased reproductive success. This is interesting, and calls into question exactly what UV tail chroma is communicating at the golf course, given that birds here are overall more highly ornamented (Chapter II).

Conclusions

In summary, results of this research provide support for the hypothesis that habitats differing in levels of human disturbance subtly influence the relationships between hormones, behavior, and plumage ornamentation as there were significant differences among these variables and between life history stages between and within sites examined here. While results generated lend
support overall to this hypothesis, predictions for results between sites were not all supported, specifically those regarding plumage ornamentation and relationships between hormones, aggression, and reproductive success. Further examination of human modified environments differing in levels of modification should be further explored in wild populations, accounting for realistic selection pressures, and examining ecologically relevant measures of physiology and reproductive success.
CHAPTER IV – FEMALE RESPONSE TO CONSPECIFICS: DIFFERENCES IN HORMONES AND AGGRESSION ACROSS NESTING IN A GOLF COURSE LIVING POPULATION OF EASTERN BLUEBIRDS

Introduction

Maladaptive impacts of anthropogenic disturbance on behavior (reviewed in Clotfelter et al. 2004; Carere et al. 2010) and physiology (reviewed in Colborn et al. 1993; reviewed in Frye et al. 2012; Ottinger et al. 2009) of non-target organisms have been well documented in a number of species. More recently, focus has shifted to include behavioral adjustments in examining responses to noise, light, and environmental pollution, in addition to human presence, traffic, and human-made structures (Barber et al. 2010; reviewed in Sol et al. 2013). Many species live and survive in proximity to humans and a current area of disturbance research focuses on what allows some species to coexist easily with humans (Finney et al. 2005; Bisson et al. 2009).

Female birds are ideal for studies investigating physiological impacts of disturbance due to sensitive reproductive physiology and related behaviors impacting fitness. Additionally birds exhibit distinct life history strategies and mating ecologies resulting in predictable and testable relationships between hormones, behavior, and physiology. Last, many bird species are drawn to human dominated environments during breeding, when human activities can impact fitness in an ecologically relevant manner. Approaches to examining disturbance are refocusing on site-specific, demographic-specific, and
population-specific impacts (reviewed in Gill, 2007), and this dissertation research uses these methods to explore avian female physiology and behavior in this chapter.

**Hormonal Regulation of Breeding and Behavior**

The hypothalamic-pituitary-gonadal (HPG) axis, and its resultant hormone products, is responsible for regulating courtship and reproductive behaviors in both birds and mammals (reviewed in Adkins-Regan, 2005; Nelson, 2011). Hormones produced by these structures are highly conserved across vertebrates and involved in responses to fitness-related environmental and physiological cues (reviewed in Adkins-Regan, 2005). Reproduction in photoperiodic passerine birds is highly endocrine regulated, with increasing photoperiod stimulating testicular growth in males and ovarian growth in females; gonadotropin-releasing hormone (GnRH) from the hypothalamus assists in this seasonal gonadal regulation (Dawson et al. 1985; Parry et al. 1997). This, however, is not the case for all songbirds as some irruptive breeders have been shown to respond to food or water resources as opposed to day length (Hahn, 1998; MacDougall-Shackleton et al. 2006). In female birds, ovulation and egg laying are also under endocrine control, influenced by a surge of lutenizing hormone (LH; produced via HPG-axis), and eggs are normally laid within a day of this surge (reviewed in Johnson, 2011).

Developmentally, exposure to steroid hormones, testosterone (T) and estrogen (E$_2$) at the embryonic stage, may differentially impact normal sexual and
social development in adults (reviewed in Johnson, 2011; reviewed in Deviche et al. 2011). Additionally, study in chickens (Gallus gallus) show active aromatase (AROM; converts T to E₂) by day 5 of development in females but not in males (Nomura et al. 1999). While reproduction in songbirds is the result of suites of temporally coordinated physiological events, this research will focus mainly on T as it is essential to appropriate follicular maturation, egg production, organizational embryonic development, and is influential in adult reproductive function, courtship, aggressive, and territorial behaviors in females (reviewed in Adkins-Regan, 2005; reviewed in Johnson, 2011).

Exogenous T. Studies that use exogenous application of androgens (implants) reveal mechanisms mediating T and aggression in males, demonstrating influences of T on many behaviors impacting fitness (Ketterson et al. 2009). Similar implant studies in females reveal information about covariation between androgens, stress hormones, immunity, aggression and parental care behaviors (reviewed in Cain and Ketterson, 2012). Females with experimentally increased T show increases in aggressive interactions and behavior, reductions in cell mediated immunity (Zysling et al. 2006), poor nest defense, lower reproductive success (O'Neal et al. 2008), increases in baseline hypothalamic-pituitary axis (HPA) activation (e.g. increased corticosterone, CORT) (Sandell, 2007), and trade-offs between increased conspecific aggression and decreased maternal behavior (Rosvall, 2013). Some species show induction of male courtship behaviors and brain structures (e.g. song and song control system)
(Rouse et al. 2015), while others display no aggression elevation or performances of male-typical social or sexual behaviors (Lahaye et al. 2012). While implant studies are informative, there is a more complex relationship between aggression and endogenous T in females. Implant study interpretation must be assessed carefully as these studies demonstrate possible impacts of elevated T, not definitively what occurs when T naturally elevates in the wild.

**Endogenous T.** The complex relationship between aggression and endogenous T has been extensively studied in female dark eyed juncos (*Junco hyemalis carolinensis*). Females capable of producing higher T (in response to a GnRH challenge) respond more quickly to a simulated territorial intrusion (STI) during nesting (Cain and Ketterson, 2012). However, endogenous T does not increase significantly during intra-sexual interactions between female juncos (Jawor et al. 2006). Females sampled for expression of androgen and E₂ receptors and AROM in the brain (specifically, song control nuclei, amygdala, and hypothalamic nuclei) after STI show correlations between expression of genes controlling physiological T production and response and aggressive behavior, while aggression was unrelated to circulating T (Rosvall et al. 2012). Behavioral assays reveal that highly aggressive females accrue benefits leading to higher reproductive success (Sandell, 1998; Jawor et al. 2006; Cain and Ketterson, 2012) but conspecific, female-female aggression is unrelated to the quality of males mated to females (Cain, 2014). Female response to social challenges also differs, with highest aggression elicited from a predator mount,
less to female intruders, and even less to male intruders during incubation (Cain et al. 2011).

**Physiological Response to Aggression.** Aggressive responses of females to STIs across songbird species, and in relation to T, vary as some territorial females do not always respond with increases in T or aggressive behavior (Canoine and Gwinner, 2005; Jawor et al. 2006; DeVries et al. 2015) while some increase CORT and decrease androgens (Navara et al. 2006) or elevate levels of LH and E₂ (Schwabl, 1992). Responses to territory intrusion can be influenced by mating status, body size, and condition (Jonart et al. 2007) and increases in conspecific density can result in increases in aggression (Bhardwaj et al. 2015). Notably, increased female aggression in response to STIs before and during egg laying can result in higher concentrations of yolk T in eggs laid post-intrusion (Wittingham and Schwabl, 2001; Navara et al. 2006) and even non-aggressive context increases in circulating T can lead to increases in egg yolk T (Jawor et al. 2007). Variation in egg yolk T can have a wide range of effects on developing offspring (reviewed in Groothius et al. 2005; reviewed in von Engelhardt and Groothuis, 2011) and as a result of studies such as these, female-specific, adaptive, functional mechanisms of breeding season relationships between aggression and T have received more examination.

**Maternal and Yolk Androgens and Impacts on Selection for T and Aggression levels:** Mechanistic facilitation of female aggression (especially in relation to steroid hormones) is of interest in songbirds due to ‘maternal effects’
(e.g. relationships between egg contents, genotype, and environment) (reviewed in Groothius and Schwabl, 2008; reviewed in von Engelhardt and Groothuis, 2011). Yolk steroid hormones are maternally derived; female hormone levels, in general and those deposited into eggs, are influenced by both female environment and physiological condition. Females can mitigate responses of future generations, e.g. match offspring phenotype to maternal environments where survival is variable (reviewed in Groothius and Schwabl, 2008; reviewed in von Engelhardt and Groothuis, 2011), and this could impose selection on functional facilitation of female aggression. The interplay between potential selection for low T to avoid maladaptive maternal effects and the need for T to facilitate certain breeding behaviors makes for an intriguing association between selective pressures on a single hormonal stimulus.

**Testosterone and Aggression in Females**

When examining breeding season aggression and androgens of female birds, researchers use similar theoretical and mechanistic approaches to that of studying male birds; the similarity in male and female genomes often results in coevolution of phenotypic traits between the sexes, especially in cases where species experience strong sexual selection (Ketterson et al. 2005; Rosvall, 2013). Genetic correlations between the sexes coupled with strong selection for annual fluctuation in T in males may explain similar annual peaks in T among females (Ketterson et al. 2005; Møller et al. 2005). Genetic correlations, however, do not adequately explain variation in aggression and T in all songbird
species, as there is also evidence for direct selection on females (Siefferman et al. 2005). For example, ornaments signal dominance and are positively associated with baseline T in female, but not male, American goldfinches (Spinus tristis; Pham et al. 2014). Conversely, circulating endogenous T is often related to STI elicited aggression in males but not females, while individual variation in behavioral aggression could be predicted by gene expression for androgens, E₂ receptors, and AROM levels in both sexes (Rosvall et al. 2012).

Considering T’s facilitation of aggression in males and females, mechanisms facilitating aggression in males may explain behavior in females but researchers must also consider the female-specific biological costs of high T (Rosvall, 2013). Mechanistic control of female aggression could be achieved by lower levels of T in females, and/or, increased or reduced sensitivity to T in certain target tissues (e.g. reduced in areas associated with maternal care, increased in areas associated with aggression) (Rosvall, 2013). Females may also regulate hormone levels independent of the HPG-axis (reviewed in von Engelhardt and Groothuis, 2011, Rosvall et al. 2012). This may allow females to avoid systemic costs of elevated T such as reduced offspring condition (reviewed in von Engelhardt and Groothuis, 2011) or masculinized reproductive behaviors (Ketterson et al. 2005).

Aggression, Stress, and Anthropogenic Disturbance

The complex and sensitive relationships between female aggression, T, and maternal effects are ideal for examination in relation to anthropogenic
disturbance. Discussion of T and disturbance, however, often necessitates discussion and inclusion of CORT, a hormone sensitive to disturbance and stressful events (reviewed in Adkins-Regan, 2005). In species where males have high T, or are exposed to high T via implants, the HPA-axis may also activate and elevate CORT (Schoech et al. 1999; reviewed in Adkins-Regan, 2005).

Aggressive interactions amongst individuals are inherently stressful and CORT facilitates similar adaptive physiological responses in these scenarios similar to T (reviewed in Adkins-Regan, 2005). Responses to disturbance in birds are often examined in the context of the glucocorticoids (GCs; CORT in birds) (Partecke et al. 2006; Bonier et al. 2012; Potvin and MacDougall-Shackleton, 2015), with highly variable results. Baseline CORT levels, always present in the blood, provide increased access to energy stores, as both daily and annually energetic demands fluctuate; termed predictive homeostasis (Romero, 2004; Nelson, 2011).

Because stress results from both individual perception and experience, stress at the individual level is difficult to define and explain (Romero, 2004; Nelson, 2011). Here, I define stress as any potential disruptor of an individual’s homeostatic state. When this occurs, an individual undergoes a stress response initiated by epinephrine and both facilitated and mediated by (potentially drastically increased) CORT, termed reactive homeostasis (Romero, 2004). If stressors are prolonged, the response becomes maladaptive, with elevated, prolonged CORT affecting overall attempts at reproduction, reduced production
of young, and reduced nestling provisioning (reviewed in Adkins-Regan, 2005; reviewed in Bruener, 2011).

Increasing anthropogenic disturbance has caused the transition of environments from rural to urban in nature, spurring investigation of physiological responses of birds to such events. Generally, urbanization appears to select for altered stress responsiveness overall (Partecke et al. 2006; reviewed in Bonier, 2012). Colonization of species into previously unavailable habitat may be driven by more aggressive individuals (Duckworth, 2008), and in general, within species, urban populations tend to be bolder and more aggressive (Scales et al. 2011; reviewed in Bonier, 2012). Physiological mechanisms (and hormone involvement) behind such behavioral adjustments are not well studied and require further investigation.

The *risk-disturbance hypothesis* (reviewed in Frid and Dill, 2002) assumes that animals perceive humans as predators and alter energy budgets (e.g., CORT responses) due to chronic stress. Additionally, this hypothesis predicts that aggressive responses to disturbance events (and their physiological correlates) will increase with increasing perceived predation risk and stage of reproductive investment (Frid and Dill, 2002). Predictions generated from this hypothesis address population level responses; both between and within sites that vary in disturbance, birds residing in more disturbed areas are predicted to respond to aforementioned disturbance events with increasing aggression and CORT (Frid and Dill, 2002). Between sites, higher CORT would be expected in a
more disturbed environment, and within sites, CORT is expected to increase across the nesting cycle along with aggression (Frid and Dill, 2002). While T has the potential to elevate in this manner, there is the chance that T may not follow the same pattern in females as it does in males given potential maladaptive impacts of maternal effects (reviewed in von Engelhardt and Groothuis, 2011).

Eastern Bluebirds

_Ultraviolet Plumage in Females._ In the early 20th century, the eastern bluebird (_Sialia sialis_) experienced reduced population numbers in relation to decreased naturally available nesting cavities (Gowaty and Plissner, 1998), and nest boxes provided by birding enthusiasts facilitated bluebird colonization of human dominated environments while increasing population numbers.

Eastern bluebirds are socially monogamous, bi-parental, and sexually dichromatic (for full detail, see Chapter I). Males compete aggressively for access to territories (and mates), and the UV chroma of blue plumage predicts ability to secure limited nesting sites and elicits more aggression from same-sex conspecifics (Siefferman and Hill, 2005; Mercadante and Hill, 2014), however, function of similar ornamentation in females is still relatively unknown. Plumage coloration is potentially a signal of reproductive quality in female bluebirds as UV blue color is sensitive to food manipulation (Siefferman and Hill, 2005a). In an Alabama population, UV blue female coloration correlates with earlier egg laying dates, offspring provisioning rates, and number of offspring fledged (Siefferman...
and Hill, 2005a). In an Oklahoma population, UV blue coloration is associated with earlier laying dates (Grindstaff et al. 2012).

**Female bluebird CORT, T, and aggressive responses.** Exploration of female bluebird hormones is occurring more frequently, revealing relationships between steroid hormones and UV plumage, altered stress responses, aggression, conspecific density, and yolk deposition into eggs. For example, females displaying increased UV blue coloration have both higher CORT and lower T in an Oklahoma population (Grindstaff et al. 2012). Stress responses in female bluebirds may be flexible, as they initially increase CORT to capture-restraint protocols but repeated exposure can result in habituation (Lynn et al. 2010). Lastly, both eastern and western bluebirds have been shown to exert maternal effects on future offspring via altered yolk steroid deposition in response to both simulated (Navara et al. 2006) and natural territory intrusion and variation in box density (Duckworth, 2008; Duckworth et al. 2015). However, although females that experience STIs during egg yolking deposit higher levels of T in eggs, circulating T was significantly lower suggesting females may use this mechanism to prevent high T from disrupting reproduction (Navara et al. 2006).

**Ideal Species for Anthropogenic Study.** Bluebirds prefer to nest in open, short grass habitats interspersed with trees and snag (Jones et al. 2014), which can be limited in human environments (see Gillespie, Chapter 1 for full detail). Golf courses typically have open short grass habitat, interspersed with small clusters of trees and often bordered by a forest edge, potentially attracting high
quality individuals to these environments. Previous investigation of bluebirds breeding on golf courses show variable results, with some populations delaying reproduction (Stanback and Seifert, 2005) and others increasing reproductive productivity but with lower probability of nest survival prior to hatching (LeClerc et al. 2005). Anthropogenic noise results in altered self-maintenance behavior and altered song behavior (Kight and Swaddle, 2007; Kight and Swaddle, 2015), again showing potentially adaptive responses to disturbance. Lastly, bluebirds experience increased reproductive output at intermediate levels of human disturbance (Kight et al. 2007), however, breeding in noisier environments results in reduced productivity (e.g., smaller broods) (Kight et al. 2012).

Justification and Hypothesis

In this research, I investigate relationships between female-female conspecific aggression, T, CORT, ornamental plumages, and reproductive measures in female bluebirds. I analyze populations from two sites, a golf course and rural military base, differing in level and type of human modification and activities (for full detail, see Chapter I). Golf course males and females are significantly more ornamented for both breast and tail ornaments (Chapter II). Populations will be investigated for flexible, phenotypic variation in behavioral aggression, hormonal responses, and ornament signaling during different life history stages. I perform simulated territorial intrusions during nest building and incubation to assess both aggression and hormonal response. My hypothesis is
that subtle differences in the relationships between hormones, plumage, and behavior in this species are influenced by increased disturbance.

**Predictions between sites**

Based on site differences (open habitat, nest box density, and chronic human activity, and land management practices; Chapter I) and the *risk-disturbance hypothesis* (Frid and Dill, 2002), I predict that females at the golf course will 1) Respond to intrusions with increased aggression (Frid and Dill, 2002; reviewed in Bonier, 2012; Bhardwaj et al. 2015), 2) Display lower levels of T (reviewed in von Engelhardt and Groothuis, 2011) and higher levels of CORT (reviewed in Frid and Dill, 2002) due to anti-predator strategies associated with nest defense and potential relationships among T and CORT as discussed in Adkins-Regan (2005), 3) Experience delayed egg laying due to variation in stress physiology, nesting resources, increased human disturbance and 4) Show different relationships among hormones, behavior, plumage and reproductive indices within sites compared to females breeding in the more rural and natural habitat (supported by findings reviewed in both Frid and Dill, 2002; Carere et al. 2010).

**Predictions within sites**

Within sites, I predict more ornamented individuals (e.g. expressing darker melanin breast pigment and greater UV tail chroma) will 1) Display higher CORT and lower T as in Grindstaff et al. (2012), 2) Respond to intrusions with increased aggression (Mercadente and Hill, 2014), and 3) Lay eggs earlier (Siefferman et
al. 2005; Grindstaff et al. 2012). Females living at the golf course (e.g., the more disturbed population), under the risk disturbance hypothesis (reviewed in Frid and Dill, 2002), will experience increases in 4) Response T, 5) Response CORT, and 6) Response aggression from nest building to incubation (use of ‘response T/CORT’ to differentiate results/discussion regarding hormones across nesting within sites as opposed to levels compared between sites; measure presented in still the same, e.g. hormone response to STI).

Methods

Study Sites

High Disturbance Location. The high human disturbance population of bluebirds breeds in nest boxes at a golf course in Hattiesburg, MS (31 20.9’ N, 89 22.6’ W). There is a significantly higher percent of open habitat and water at this site, and nest boxes are more densely located (see Chapter I). The turf is maintained daily, grass is kept short, and pesticides are applied regularly (see Chapter I, Table 1 for full detail); boxes here experience significantly higher human traffic and are more densely located (Chapter I). I monitored 45 boxes at this site. Low Disturbance Location: The low disturbance site is Camp Shelby Joint Forces Training Center (Camp Shelby, Hattiesburg, MS; 31.19’ N, 89.20’ W). The large base is rurally located on the outskirts of Hattiesburg (542,278,761 square meters). Human presence is infrequent, some boxes do experience significantly higher vehicle traffic, boxes are less densely dispersed, and this site
has a higher percent of roads and buildings (though not near monitored boxes) (Chapter I). I monitored 120 nest boxes at this site.

*Bird Capture and Monitoring*

Bluebirds begin prospecting for territories and mates in February and March and breed through August. Bluebirds in southern US populations may produce up to 3 successful broods in some populations. I captured birds through the breeding season from 0400-1200h using mist nets or box traps and measured mass, tarsus length, wing chord, and tail length. Additionally, samples from both breast and tail plumages were collected upon initial capture (see Chapter II for full detail). I banded birds with a USFWS identification band (subpermit #23479-C) and a unique combination of color bands. In 2013, I monitored birds at the golf course, in 2014 birds at Camp Shelby, and in 2015, both sites. I monitored boxes daily to record nest building and every other day for initiation of egg laying. Once eggs were laid and/or nestlings hatched, I monitored survivorship of nests every 3 days. All field work was approved by the University of Southern Mississippi’s Institutional Animal Care and Use Committee (#12042601) and Mississippi’s Department of Wildlife, Fisheries, and Parks (#0319131).

*Behavioral Assays*

I performed two, same sex, conspecific simulated territorial intrusions during nest building (after nesting material found) and incubation (second week of incubation). A female intruder caught elsewhere on site was placed in a small
wire cage 0.3m from the nest-box of the focal female. A speaker broadcasted bluebird song and chatter to begin intrusions. I recorded latency time until each adult arrived (within 40m of the nest box) followed by time of the first attack, and the total number of times the focal female 1) attacked, 2) dove, 3) took flights to nest box, and 4) times the bird landed on top of the intruders cage. After 10 min, I attempted to capture the focal female. I continued playing bluebird vocalizations up to 30 min after STI and behavioral observation. Upon capture, I took blood from the brachial vein within 3 min of capture. Blood was stored in a cooler until centrifuged and plasma was stored at -20°C.

**Hormone Assays**

For both T and CORT assays, when I collected multiple plasma samples from single individuals, I analyzed them on the same plate, but sample placement was random throughout plates. Intra-assay and inter-assay variation were calculated by randomly placing standards of known T and CORT concentrations throughout the plate, with T standards of known concentration and CORT standards provided from northern bobwhite (*Colinus virginianus*) homogenized plasma, respectively.

*Testosterone.* I conducted analyses using an enzyme linked immunosorbent assay (ELIZA; Enzo Life Sciences) following methods outlined in Jawor (2007). I extracted hormones using diethyl-ether (3x), resuspended extracts, and diluted them with ethanol (50µl) and kit supplied assay buffer (300µl). Prior to extraction plasma samples were combined with tritiated T (2000
cpm, H^3T, PerkinElmer) to allow for calculation of hormone recovery percentages (mean= 72%). I then measured T via ELIZA in duplicate 100µl quantities using kit supplied antibodies sensitive to T. I calculated concentrations of T and corrected for incomplete recoveries (Microplate Manager, Bio Rad Laboratories; H1 Synergy Reader™, BioTek, Winooski, VT). I calculated intra-assay and inter-assay variation by randomly placing standards throughout the plate; intra-assay variation was 1-24% while inter-assay variation was 6%.

**Corticosterone.** I conducted analyses using an enzyme linked immunosorbent assay (Arbor Assays, LLC) as outlined in DeVries and Jawor (2013). As with T, I extracted CORT using diethyl-ether (3x), resuspended extracts, and diluted them with kit supplied assay buffer (400µl). I combined plasma samples (10ul) with tritiated CORT (2000 cpm, H^3CORT, PerkinElmer) to allow for calculation of hormone recovery percentages (mean= 76%). I calculated concentrations of CORT and corrected for incomplete recoveries (Microplate Manager, Bio Rad Laboratories; H1 Synergy Reader™, BioTek, Winooski, VT). Intra-assay variation was 1-17% while inter-assay variation was 24%. Inter-assay variation inflation is due to the use of multiple plates from multiple kits.

**Plumage Analysis**

For breast plumage, mean brightness (percentage of light reflected; see Chapter II) was calculated using a S2000 spectrometer (visible range; Ocean Optics, Dunedin, FL) was used to take reflectance measurements from melanin pigmented breast feather samples. For UV tail plumage, samples were sent to
Dr. Lynn Siefferman (Appalachian State University) for analysis using also a S2000 spectrometer (UV range; Ocean Optics, Dunedin, FL) was used to take measurements (3 replicates) from the identical areas of each tail feather, replicate reflectance measures were averaged and UV chroma (also called saturation or spectral purity) was calculated (see Chapter II for full detail). Different spectrometers used were specifically tuned to wavelengths of interest (e.g. visible and UV spectra) for accuracy of reflectance measures (reviewed in Andersson and Prager, 2006; for full detail see Chapter II).

Statistics

I used SPSS (version 23.0) for data analysis. Data tested for normalcy using Shapiro-Wilk were not normally distributed. I either log transformed or standardized for year (using z-scores) for use of parametric tests. Both independent and paired samples t-tests were used to examine variables between sites and life history stages, respectively. Variables were then correlated separately within sites using raw data and Spearman’s Correlations. Results presented are for site variation in STI elicited T, CORT, aggression, differences in dates first eggs are laid, and within site correlations of these variables to both melanin and UV plumages. Raw data (mean, standard deviation, and range) for T, CORT, and aggression are also presented as standardization for year did not normalize distributions of all variables and because standardization for year can often result in loss of biologically meaningful information (Lynn Siefferman, pers. comm.). Benjamini-Hochberg step-wise procedure (Benjamini and Hochberg,
1995) was used to sequentially reduce the number of comparisons to control for false discovery rates when multiple comparisons are used (when necessary significance after corrections are presented in tables; false discovery rate was set to 10%) (McDonald, 2014).

Results

Handling and Capture

On average, birds were bled within 3 min of capture (mean=2.13, N=96, SD=0.997) and there was no effect on hormones of time elapsed until capture (post-behavioral observation) or time elapsed during blood sample collection (post-capture in mist net) (Table 14).

Table 14

Handling and Capture Effects on Hormones

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Golf Course</th>
<th>Campy Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_s$</td>
<td>$p$</td>
</tr>
<tr>
<td>$T*Bleed$</td>
<td>0.073</td>
<td>0.696</td>
</tr>
<tr>
<td>$CORT*Bleed$</td>
<td>0.071</td>
<td>0.711</td>
</tr>
<tr>
<td>$T*Capture$</td>
<td>-0.100</td>
<td>0.591</td>
</tr>
<tr>
<td>$CORT*Capture$</td>
<td>-0.301</td>
<td>0.106</td>
</tr>
</tbody>
</table>

Bleed=time elapsed from net removal to cessation of blood sampling; Capture=time elapsed from cessation of behavioral observation to bird’s capture in mist net.

Annual Variation in Hormones, Behavior, and Reproductive Indices

First egg dates varied by year within sites. Measures of T (Figure 20) and CORT (Figure 21) varied by year within sites while attack number did not vary between years (Table 15). Variables displaying annual variation are standardized for year in future analyses.
### Table 15

**Annual Variation within Sites: Hormones, Behavior, First Egg Date**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Golf Course</th>
<th></th>
<th>Camp Shelby</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Year</td>
<td>N</td>
<td>F(df)</td>
<td>p</td>
</tr>
<tr>
<td>T</td>
<td>2013</td>
<td>15</td>
<td>5.062 (1,31)</td>
<td>0.032*</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CORT</td>
<td>2013</td>
<td>16</td>
<td>26.635 (1,29)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aggression</td>
<td>2013</td>
<td>26</td>
<td>0.817 (1,58)</td>
<td>0.370</td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Egg</td>
<td>2012</td>
<td>15</td>
<td>37.53 (2,76)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Date</td>
<td>2013</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2015</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N = sample size; F(df) = test statistic for ANOVA; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparison.

**Figure 20.** Annual Variation in Female T
Site Comparisons of Hormones, Aggression, First Egg Dates

There were no significant differences between sites for females in T or CORT responses to conspecific intrusions (Table 16). Golf course females respond to intrusions with significantly less aggression than Camp Shelby
females (Table 16, Figure 22). Lastly, golf course females lay eggs significantly later than Camp Shelby females (Table 16).

Table 16

Site Comparisons for T, CORT, Aggression, and First Egg Dates

<table>
<thead>
<tr>
<th>Variable</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
<th>Independent Samples t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>T</td>
<td>0.11</td>
<td>1.04</td>
<td>32</td>
</tr>
<tr>
<td>CORT</td>
<td>-0.09</td>
<td>0.89</td>
<td>30</td>
</tr>
<tr>
<td>Aggression</td>
<td>0.33</td>
<td>0.65</td>
<td>59</td>
</tr>
<tr>
<td>First Egg Date</td>
<td>0.24</td>
<td>0.98</td>
<td>77</td>
</tr>
</tbody>
</table>

M=mean; SD=standard deviation; n=sample size; means and standard deviations presented in this table are for data standardized for annual variability; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.
Figure 22. Female Aggression across the Nesting Cycle

Number of Attacks toward intruder= total attack number within 10 min observation period. Raw data is used in the graph. The golf course is represented by light gray stars and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, the open circles are beyond the 10th and 90th percentiles, and stars are outliers. Outliers are included as golf course females attack so infrequently that any attacking golf course female is considered an outlier.

Site Variation in Raw T and CORT

Variation in raw T concentrations in golf course females show higher mean raw T (Figure 23) than Camp Shelby females and similar mean raw CORT concentrations between sites (Table 17). Additionally, golf course females produce almost 3 times the range of T and almost double the range of CORT in comparison to Camp Shelby females (Table 17). Raw data for aggression shows
that Camp Shelby female’s mean attack number is 3 times that of golf course females and double the range (Table 17).

Table 17

*Site Variation in Raw T and CORT*

<table>
<thead>
<tr>
<th>Response</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
</tr>
<tr>
<td>T</td>
<td>1.07</td>
<td>1.402</td>
</tr>
<tr>
<td>CORT</td>
<td>29.81</td>
<td>21.94</td>
</tr>
<tr>
<td>Aggression</td>
<td>10.03</td>
<td>25.72</td>
</tr>
</tbody>
</table>

M=mean; SD=standard deviation; n=sample size; means, standard deviations, and ranges presented in this table are for raw data

![Graph](image)

*Figure 23. Mean T Responses*

Raw data is used in graph. The golf course is represented in white, open bars and Camp Shelby is represented in dark grey bars. The line the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and the circles are beyond the 10th and 90th percentiles.
Conspecific Intrusions: Variation between Life History Stages within Sites

Results of paired samples \( t \)-tests show significant increases in \( T \) (Table 18, Figure 24) and CORT (Table 18, Figure 25) across intrusion types within each site while aggression decreases at the golf course and increases at Camp Shelby from nest building to incubation intrusions (Table 18, Figure 26).
Table 18

*Nest Building & Incubation: T, CORT, Aggression within Sites*

<table>
<thead>
<tr>
<th></th>
<th>M</th>
<th>SD</th>
<th>N</th>
<th>T</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Golf Course</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>1.33</td>
<td>1.06</td>
<td>32</td>
<td>7.12</td>
<td>31</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>CORT</td>
<td>1.52</td>
<td>0.98</td>
<td>29</td>
<td>8.55</td>
<td>29</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Aggression</td>
<td>1.14</td>
<td>0.86</td>
<td>58</td>
<td>10.20</td>
<td>58</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td><strong>Camp Shelby</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>1.57</td>
<td>1.08</td>
<td>53</td>
<td>10.61</td>
<td>53</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>CORT</td>
<td>1.47</td>
<td>1.11</td>
<td>48</td>
<td>9.14</td>
<td>47</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Aggression</td>
<td>0.66</td>
<td>0.95</td>
<td>68</td>
<td>5.79</td>
<td>67</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

M=mean; SD=standard deviation; n=sample size; means and standard deviations presented in this table are for data standardized for year; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.

*Figure 24. Female T across the Nesting Cycle*
Raw data is used in the graph. The golf course is represented in white, open bars and Camp Shelby is represented in dark grey bars. The line the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and the circles are beyond the 10th and 90th percentiles.

Figure 25. Female CORT across the Nesting Cycle

Raw data is used in the graph. The golf course is represented in white, open bars and Camp Shelby is represented in dark grey bars. The line the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and the circles are beyond the 10th and 90th percentiles.
Figure 26. Female Aggression across the Nesting Cycle

Raw data is used in the graph. The golf course is represented by light gray stars and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, the open circles are beyond the 10th and 90th percentiles, and stars are outliers. Outliers are included as golf course females attack so infrequently that any attacking golf course female is considered an outlier.
Figure 27. T and Aggression in Females at both Sites

Raw data is used in the graph. The golf course is represented by open circles and Camp Shelby is represented in dark grey circles.

Relationships between Variables within Sites

There were no significant correlations within sites for ornamental plumages (both UV chroma and melanin pigmented), CORT, T, attack number, or first egg dates (Table 19).

Relationships between Variables Overall (Sites Combined)

Response variables, T and attack number (Figure 27), were plotted against one another and variable distribution was categorized (presence/absence) for location on graphing plane. Values were then used in a Chi-Square test of independence to examine the relationship between T and aggression. The relationship between these variables was significant $X^2$ (1,
N=85)=85.00, p<0.0001 (Figure 27), demonstrating a non-random distribution of points. Although females with low T exhibited a variety of aggressive behaviors, females with high T were not aggressive (Figure 27).

Table 19

*Relationships between Variables within Sites*

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
</tr>
<tr>
<td>UV*Melanin</td>
<td>0.201</td>
<td>0.225</td>
</tr>
<tr>
<td>UV*CORT</td>
<td>-0.381</td>
<td>0.05</td>
</tr>
<tr>
<td>UV*T</td>
<td>-0.427</td>
<td>0.02</td>
</tr>
<tr>
<td>UV*First Egg</td>
<td>-0.318</td>
<td>0.04</td>
</tr>
<tr>
<td>UV*Attack</td>
<td>0.123</td>
<td>0.380</td>
</tr>
<tr>
<td>Melanin*CORT</td>
<td>0.120</td>
<td>0.670</td>
</tr>
<tr>
<td>Melanin*T</td>
<td>-0.208</td>
<td>0.393</td>
</tr>
<tr>
<td>Melanin*First Egg</td>
<td>0.221</td>
<td>0.224</td>
</tr>
<tr>
<td>Melanin*Attack</td>
<td>0.116</td>
<td>0.474</td>
</tr>
</tbody>
</table>

Raw data is used in this analysis; rs = Spearman’s rho, correlation coefficient; N= sample size; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.

Discussion

*Summary of Results*

*Between Sites.* Research here partially supports the hypothesis that differing levels of human disturbance impact relationships between hormones, behavior and physiology of female bluebirds. In the more intensively human-impacted environment (golf course) I found mean differences in timing of breeding and aggression when compared to the rural site. Golf course females were significantly less aggressive during simulated territorial intrusions (Figure 26) and initiated egg laying significantly later in the season. However, females at
the golf course exhibited raw T (Figure 23) and CORT concentrations that were nearly three times and two times higher, respectively, than females at the less disturbed site (Camp Shelby). Unexpectedly, there were no significant relationships between hormones, behavior, or ornamental plumages within either site.

*Within Sites.* Predictions under the risk disturbance hypothesis (reviewed in Frid and Dill, 2002) are partially supported, as both golf course and Camp Shelby females significantly increase both response T (Figure 24) and response CORT (Figure 24) over the nesting cycle. Predicted aggressive behavior in golf course females was not supported, as golf course females displayed reduced aggression from nest building to incubation (Figure 26), while Camp Shelby displayed increases in aggression across nesting (Figure 26), consistent with the risk disturbance hypothesis (reviewed in Frid and Dill, 2002).

There is significant annual variation in T (Figure 20), CORT (Figure 21), and first egg dates for females at both sites. Golf course and Camp Shelby females displayed significantly higher T and CORT in 2013 than in 2015, while Camp Shelby females displayed significantly higher T and CORT in 2014 than in 2015. First egg dates are significantly later for golf course females in 2013 and Camp Shelby females in 2014 in comparison to 2015 dates.

Given the T levels displayed compared to the levels of aggression, this relationship appears to be depicting what could be a constraint envelope (Figure 27), which proposes a mechanism whereby females are not physiologically
capable of significantly elevating T and expressing behavioral aggression at the same time, with this relationship being stronger at the golf course. This presents the possibility of a fascinating disconnect between behavior and hormone influences at one of the sites and potentially compensatory mechanisms that allow elevated aggression in the face of lower hormone modulators (Canoine and Gwinner, 2005; Jawor et al. 2006; Duckworth, 2008; Rosvall, 2013; DeVries et al. 2015).

**Annual Variability**

In some variables the sites here significantly differ, with the golf course habitat experiencing significantly higher levels of human activity, percent open habitat, and nest box density (Chapter I). Additionally, sites differ in both land-use and grounds maintenance practices (Chapter I, Table 1). The combination of these habitat differences could factor into results observed, as sites are close in proximity and unlikely to differ in larger, natural environmental variables (e.g., rainfall or temperature). For example, there are significant differences in both open habitat and percent water between sites (Chapter I), these habitat composition differences alone may result in dissimilarities in diversity of arthropod communities (Begon et al. 2005; Pimentel and Edwards, 1982), leading to variation in reproductive success and/or physiology.

The significant annual variation observed in T (Figure 20), CORT (Figure 21), and first egg dates is interesting. While larger environmental variables should not vary within or between sites, the variation observed in bluebirds
implies that some factors within sites may vary annually, potentially impacting reproduction. One possibility is heterospecific competition for food resources. Both bluebirds and fire ants (*Solenopsis invicta*) are insectivorous and previous research has established relationships between fire ant density and offspring sex ratios, re-nesting decisions, and reproductive success of bluebirds (Ligon et al. 2011). The red imported fire ant was quantified between sites during this work (L.M. Gillespie, unpublished data) to better understand their impact on reproductive success. Results for 2015 (when data collection occurred simultaneously at both sites) showed high abundance of this species at Camp Shelby, however, only the invasive Argentine ant (*Linepithema humile*) was found at the golf course (L.M. Gillespie, unpublished data). Argentine ants can alter arthropod diversity and abundance within the communities they invade by decreasing density of natural ant species and caterpillars (Estany-Tigerström et al. 2010) and they can be significant nest predators (Suarez et al. 2005). Golf course nests experienced increased predation via Argentine ants (compared to both Camp Shelby nests), and that these insects may also impact arthropod communities lends further credence to the hypothesis that this factor may be a part of the observed differences between sites in this work and deserves further research.

When examining annual variation, for T, there is an overall trend for golf course female T responses to STIs to be higher than Camp Shelby female responses (Figure 20). Annual variation in CORT, however, does not show a
similar, consistent trend over time as T does; levels between sites are similar for 2013 and 2014, with CORT being slightly lower in golf course females in 2015 (Figure 21). Both sites received full season investigation in 2013 and 2014 (February-July) while in 2015, both sites were only surveyed from February-May and only the earliest nesting individuals were examined for each site. This could help explain differences in annual variation in hormones, especially lower levels of CORT in 2015.

Temperatures, heat indices, and humidity levels are extreme during June and July in southern Mississippi, and desert living avian species have been shown to reduce foraging attempts in the face of extreme heat (Wolf, 2000). Corticosterone has been shown to increase when increased foraging is necessary (Wingfield and Kitaysky, 2002) as this helps mobilize energy reserves (for example, increased gluconeogenesis) necessary for increased activity (Nelson, 2011). Data from 2015 alone is presented as data was collected at both sites simultaneously, as such it is the only year in which there are no effects due to larger yearly variation between the two sites (e.g. resources). 2015 data was only collected until May, while the golf course (2013) and Camp Shelby (2014) received examination in February-August. Potentially, higher CORT levels in both populations in 2013 and 2014 could result from surveying birds during times of more intensive thermal stress, and birds surveyed in 2015 were examined earlier in the season when temperatures are milder.
Comparison between Sites for Testosterone and Corticosterone

Contrary to predictions, in response to STIs, there were no significant differences for T or CORT responses to intrusions when controlling for annual variation (Table 15, Figures 20 and 21). Mean raw T (Figure 23) is higher for golf course females while mean raw CORT is similar for both golf course and Camp Shelby females (Table 17). The range of raw CORT levels produced by individuals is roughly twice as high in golf course females (Table 17). Range of raw T production among individual golf course females is three times that of Camp Shelby females, implying golf course females are potentially physiologically capable of producing higher maximal T and CORT than Camp Shelby females. Camp Shelby females, however, may also have this capacity, and for reasons unknown, are not elevating these hormones in response to STI. To disentangle these results, challenges to both the HPG and HPA axes would be required to determine maximal T and CORT output, respectively.

Comparisons between Sites for Aggression

Overall, golf course females are significantly less aggressive than Camp Shelby females in response to STI (Table 3, Figure 26), contrary to original predictions. Other studies of urban avian populations show increased aggression compared to rural counterparts (Scales et al. 2011; reviewed in Bonier, 2012), and this may aid in colonization of novel environments (Duckworth, 2008). Potentially, golf course females could be considered less aggressive as the population was established prior to commencement of this study and established
colonization no longer requires intensive aggressive responses, however, the same is true of the Camp Shelby population. Increases in conspecific density are also shown to impact aggression in western bluebirds (Bhardwaj et al. 2015), and given that the golf course environment has significantly higher box density, and consequently, higher conspecific density, it is surprising that golf course females differed so strikingly in aggressive responses to conspecifics.

Comparisons between Sites for First Egg Dates

Females at the golf course commence breeding (based first eggs dates) significantly later than Camp Shelby females. This is similar to other golf course nesting birds (Stanback and Seifert, 2005; Smith et al. 2005). However, this may not be a golf-course specific effect because, at some locations, bluebird populations on golf courses display increased productivity and survival (Cornell et al. 2011) or increased fecundity and productivity, but reduced survival (LeClerc et al. 2005). Due to the use of pesticides at this site (Chapter I, Table 1), and potential for delayed insect emergence as a result (Nayak et al. 2003), the population of established golf course females may have shifted to laying eggs later such that the nestling stage is more coincident with peak abundance of insect resources or in response to reduced insect resources. Alternatively, low insect abundance or diversity, resulting from differences in habitat (Humprey et al. 1999) or pesticide use (Pimentel and Edwards, 1982; Deb, 2009) could compromise female condition and cause females to delay egg laying. Unfortunately, the cause of delayed egg laying is unknown and I can only
speculate because I was unable to quantify insect abundance at the golf course site even though multiple attempts were made. This particular issue (lack of ability to quantify insects) lends support to the latter suggestion that low energy availability may be a significant part of the later egg laying date observed at the golf course. Daily energy costs of egg production can be up to 50% above resting metabolism in passerine species, and females laying eggs earliest are often in best physiological condition, e.g. they may possess higher fat stores (reviewed in Vleck and Vleck, 2011). Golf course females, maybe due to increased stress from exposure to human traffic (Chapter I) or access to reduced nutrients or caloric intake (reviewed in Breuner, 2011), may be in poorer condition than Camp Shelby females, and therefore are not physiologically able to divert energy to egg production as early.

Alternatively, higher raw T levels of the golf course females may have contributed to delayed egg laying. Indeed, previous research has shown reductions in reproductive success, fecundity, and hatching success and increases in nest abandonment when female passerines are exposed to high T or CORT in-ovo or as adults (Rubolini et al. 2007; Almasi et al. 2008; O’Neal et al. 2008). Golf course females exhibited more variable T and higher mean T compared to females at Camp Shelby (Figure 20, Figure 23). It is possible that because females at the golf course have higher T (during nest building, discussed below), this could also delay egg laying. Golf course females also breed at higher densities and thus high T could be adaptive for females for
current competitiveness. If females respond to frequent, aggressive interactions with increased T generally (compared to baseline T), this could equate to chronic, high T exposure resulting in delayed first egg dates.

*Predictions under Risk Disturbance Hypothesis: T and CORT*

Females at both sites show higher T and CORT during incubation STIs compared to nest building, consistent with predictions supported by the risk-disturbance hypothesis (reviewed in Frid and Dill, 2002). Females at the golf course, however, experience a much more drastic increase in T from nest building to incubation than do Camp Shelby females (Figure 24).

Prior to ovulation, increased levels of LH from the pituitary and progesterone (P₄) from the ovary are produced, and higher P₄ is related to higher StAR (steroid acute regulatory) protein levels which, essentially, determine eventual production of steroid hormones (reviewed in Vleck and Vleck, 2011). As both LH and P₄ are required for ovulation and growing follicles produce higher levels of P₄, and P₄ is also used as substrate for T production, increased circulating T during nest building is certainly possible (Adkins-Regan, 2005; reviewed in Vleck and Vleck, 2011), which is why golf course females drastic increase in T to incubation appears odd in comparison to Camp Shelby female’s less severe but significant T increase.

Displaying increased T or CORT responses to STIs during the last week of incubation would not result in maladaptive maternal effects (reviewed in von Engelhardt and Groothuis, 2011) as eggs are already laid. This potentially frees
the HPG axis to elevate T in response to intruders who might interfere with
nesting attempts or try to take over nest boxes (frequently a limited resource).
Higher CORT response during incubation may be adaptive e.g. upcoming
increased energy expenditure for provisioning (Bonier et al. 2009; Bonier et al.
2011), as female European starlings (*Sturnus vulgaris*) show elevated CORT in
preparation for increased investment in second broods (Love et al. 2014). This
provides further support for results here that females may increase CORT
response to aggression during late incubation as significant energy resources,
time, and physiological effort have already been invested in the reproductive
attempt.

Maladaptive Impacts of High T. Documented results of implanted T reveal
that exposure to prolonged, elevated T negatively impacts parental care, nest
defense, aggression, and nest success in females (O'Neil et al. 2008; Cain and
Ketterson, 2012). Elevated, prolonged T exposure during the last week of
incubation may impact nestling attendance or provisioning. An acute T increase
in response to intrusion may facilitate fitness-dependent aggressive responses at
the transitory expense of maternal care (Rosvall, 2013) and a temporary yet
reduced bout of incubation has less severe consequences than complete territory
loss. Alternatively, transitory T increase in response to STI could be mitigated by
increased or reduced sensitivity to T via altered receptor number or distribution in
target tissues (Rosvall, 2013). Females may also regulate hormone levels
(independent of HPG-axis activation) via altered expression of enzymes essential
to production of steroid hormones (Rosvall et al. 2012), or via differences in hormone metabolism or catabolism (Rosvall, 2013), avoiding systemic costs of elevated T. Experimental T manipulation in this species would aid adaptive explanations of both late incubation T elevation in response to STI and lack of covariation between aggression and T found in this research.

*Predictions under the Risk Disturbance Hypothesis: Aggression*

Females often express elevated T seasonally or annually, and potentially both genetic correlations with males and/or selection acting directly on females may shape relationships between T and aggression (Rosvall, 2013). Research here demonstrates partial support for increases in aggression across nesting predicted by the risk disturbance hypothesis (reviewed in Frid and Dill, 2002), though not in the population predicted. Camp Shelby females respond to STIs with significantly higher aggression, as predicted for golf course females, during incubation compared to nest building while golf course females, contrary to predictions, decreased aggressive responses during incubation (Figure 26).

While increases in aggression by Camp Shelby females corresponded with increases in levels of both T and CORT across nesting, hormones and aggression are not correlated. Golf course females are significantly less aggressive overall, and display decreased aggression across nest building and incubation intrusions, contrary to predictions, but also with no correlation between hormones and aggression. The response among Camp Shelby females (increased aggression) is similar to an implant study in which control females
increased predator-directed aggression across the nesting stage while high T females did not (O’Neal et al. 2008).

Visualizing data reveals T and CORT appear to increase in concert with aggression, however, for each hormone, a large subset of females did not attack, and hormone titers for these individuals range between the lowest and highest values (Figure 27). Preliminary investigation into this relationship shows a significant deviation from random in the pattern of T and aggression, suggesting females, and specifically, golf course females, are physiological incapable of expressing behavioral aggression while also elevating T at high levels (Figure 27). This is the process by which, over time, behavioral insensitivity to T could be hypothesized to occur. Additionally, this physiological incapacity to support high T and high aggression may select for both decreased aggression and lower T levels overall as well, potentially explaining the overall lower aggression between sites when compared to Camp Shelby, and the peculiar decrease in aggression across nesting within the golf course alone (discussed above).

Population-level Perspectives

Populations examined here are less than 29.77 km apart, and therefore differences are unlikely to be due to large differences in temperature or climate variation. Similarities and differences between Alabama, Oklahoma, and North Carolina populations imply species-wide flexibility in female aggression and local adaptation to social situations. Prior to bluebird population declines and subsequent re-establishment of wider ranges of populations, bluebird aggression
and territoriality – focusing predominantly on males - was well documented (Gowaty and Plissner, 1998). Because natural breeding cavities were potentially always limited, selection for territoriality and aggression was likely strong. During times of habitat loss followed by reduced bluebird abundance, selection for aggressive phenotypes of both sexes was likely very intensive. More recently, bluebird box trails have made nesting sites more abundant but are predominantly located in human-dominated environments. Western and eastern bluebirds have very similar habitat requirements and interspecific territoriality (Marshall, 1979) and western bluebirds are capable of maternally directed and inherited flexibility in both dispersal and aggression (Duckworth, 2008; Duckworth, 2009). It is likely, therefore that dispersal and aggression are correlated traits in eastern bluebirds and these traits may likely influence the relative success of individual bluebirds in anthropogenic environments.

*Ornamentation, Hormones, Aggression, and First Egg Dates within Sites*

Contrary to predictions, there were no significant associations found within either site for relationships between ornamental plumages and aggression, T, CORT or first egg dates. This is unlike other bluebird populations studied in both Alabama and Oklahoma where females with higher UV tail chroma lay first eggs earlier (Siefferman and Hill 2005, Grindstaff et al. 2012). As golf course females display significantly higher, more ornamented UV chroma (Chapter II), it is surprising that there is no correlation to initiation of egg laying. Thus, my data
suggest that residence on a golf course can lead to breakdown of correlations between ornamentation and current reproductive output of female bluebirds.

Conclusions

Regardless of site, female bluebirds increase both T and CORT from nest building to incubation in response to conspecific aggression, consistent with predictions under the risk disturbance hypothesis (reviewed in Frid and Dill, 2002), with golf course females exhibiting a more extreme increase in T from nest building to incubation. There are adaptive physiological and contextual factors that may account for increases in both hormones. Camp Shelby females display increases in aggression, while golf course females display decreases in aggression in concordance with (but not in correlation with) increases in T and CORT across nesting.

One possibility is that these females in these populations are physiologically constrained in their ability to both produce high levels of T and expression behavioral aggression at the same time. If they do express this aggression when T is elevated, it could lead to altered energy budgets, resulting in maladaptive impacts of T. Given that females display delayed egg laying and produce smaller clutch sizes (to be discussed in Chapter 5), it may be the case that females are already experiencing negative physiological impacts of T (reviewed in Adkins-Regan, 2005; Nelson, 2011), which may be why they display significantly reduced aggression (e.g. as a mechanism to control for both elevating T and expressing aggression simultaneously), possibly to mitigate
costs of expressing any aggression. Over time, this pattern could lead to behavioral insensitivity to T given female bluebird breeding ecology. There are similar to results in males regarding lack of relationships between T and aggression, however, males did not appear to display the same distribution of T and aggressive responses as females.

Further investigation of T and aggression in different contexts in females will be valuable in continuing to assess this and other potential mechanisms responsible for results observed in this research. Continued application of the risk-disturbance hypothesis utilizing both behavior and physiological correlates to assess anthropogenic disturbance in wild populations will help to illuminate the subtle but impactful results that both level and type of disturbance may have. This research, by using a natural experiment, can suggest future approaches to understanding how anthropogenic influences shape animal physiology, behavior, and sexually selected signals. My research may also support findings of species-level variation in annual flexibility of behavioral and hormonal phenotypes, potentially dependent upon life history stage, environment, or both, and other empirical evidence supports this (Cain and Ketterson, 2012; Fokidis et al. 2011; Gill and Sealey, 2006; Gill et al. 2007).
CHAPTER V – PARENTAL CARE BEHAVIORS IN BLUEBIRDS LIVING ON A GOLF COURSE

Introduction

In bi-parental, socially monogamous passerine species, the contribution of both pair members to parental care is essential for nestling survival and influences adult fitness (reviewed in Cockburn, 2006). Bi-parental care in songbirds consists of suites of behaviors, performed by one sex or a combined effort of both including: nest construction, vigilance or egg guarding, mate provisioning, incubation and brooding behavior, nestling provisioning, attendance, sanitation, and defense of nestlings (reviewed in Cockburn, 2006). Because parental care is energetically expensive, physiological trade-offs often occur between current and future reproductive effort (reviewed in Alonso-Alvarez and Velando, 2012). These tradeoffs can lead to conflicts of interest between parents (Royle et al. 2004), as each parent will benefit from its partner contributing more to care (Lessells, 1999). If one parent decreases its effort, its partner’s response may be to increase its own care effort, but not so much that it completely compensates for the lost care (reviewed in Harrison et al. 2009).

Ornamental plumage, which is often expressed in songbirds with bi-parental care, can indicate quality of parental care (Hoelzer, 1989; reviewed in Griffith and Pryke, 2006; reviewed in Hill, 2006), genetic quality (reviewed in Mundy, 2006), or hormonal profiles of individuals (reviewed in Kimball, 2006). Because male birds often have more elaborate traits than females, studies of the
relationship between ornamentation and parental investment have generally focused on males rather than females (reviewed in Amundsen and Parn, 2006). In some species, males displaying higher quality ornaments also provide higher quality parental care, as indicated by the good parent hypothesis, (reviewed in Hoelzer, 1989; reviewed in Griffith and Pryke, 2006), also referred to as the good-parent-ornament hypothesis. However, reproductive investment may correlate with ornamentation or with individual condition in either sex (reviewed in Amundsen and Parn, 2006). For example, in female barn swallows (Hirundo rustica) the length of the outer tail feathers (a sexually selected trait in males, Møller, 1988) is positively correlated with the number of fledglings and likelihood of producing a second clutch (Møller, 1993).

Anthropogenic disturbance may disproportionately impact neurodevelopment of one sex (reviewed in Shenoy and Crowley, 2011), and as plumage ornaments signal parental care behaviors (reviewed in Griffith and Pryke, 2006; reviewed in Hill, 2006), increased disturbances may result in altered signaling mechanisms (reviewed in Shenoy and Crowley, 2011). Although avian parental behaviors are influenced by hormones (reviewed in Vleck and Vleck, 2011), and anthropogenic distrubance can influence hormones (reviewed in Bonier, 2012; reviewed in Ottinger and Dean, 2011), the combined investigation of disturbance impacts on parental care, reproductive output, and physiology are still relatively poorly studied.
The risk disturbance hypothesis posits that animals living in human-dominant areas may perceive humans as predators, and as such, animals may invest energy in anti-predator strategies causing alterations in normally observed social or parental care behaviors (reviewed in Frid and Dill, 2002). This hypothesis predicts that CORT (and likely T) may increase over the nesting cycle in more disturbed populations (reviewed in Frid and Dill, 2002). This pattern of hormone secretion in relation to aggressive behavior in females is observed on both the golf course and Camp Shelby sites to be assessed here (Chapter IV). For males, individuals at both sites showed decreased CORT (instead of increasing), and golf course males increased T across nest building and incubation intrusions while Camp Shelby males showed decreases (Chapter III). The risk-disturbance hypothesis predicts, that between populations, more disturbed populations will exhibit increased anti-predator vigilance behavior at the expense of foraging (reviewed in Frid and Dill, 2002), which could potentially lead to decreases in both adult provisioning rates to offspring and nest attendance rates. Additionally, this hypothesis predicts decreases in reproductive success in more disturbed populations (reviewed in Frid and Dill, 2002), resulting from both increased stress and altered energy budgets.

Here, two populations of eastern bluebirds (*Sialia sialis*) are examined for differences in parental care behaviors and reproductive indices. These sites (a golf course and rural military base) differ in level of human disturbance, habitat features, land-use, and human activity (Chapter I). Ornamentation differs
between sites, with both sexes at the golf course exhibiting greater ornamentation (greater UV tail chroma and darker melanin breast coloration) (Chapter II). I hypothesize that aspects of parental care behaviors and ornament-behavior relationships will differ between and within sites.

At each site, I observed parental provisioning to nestlings and nest attendance rates and recorded measures of reproductive output (time to complete nest, incubation length, clutch size, and nestling age at fledging). I hypothesize that parental investment and ornament-behavior relationships will differ between sites.

Predictions between Sites

Based on results showing increased human activity at the golf course compared to Camp Shelby (Chapter I), and the predictions of the risk disturbance hypothesis (reviewed in Frid and Dill, 2002), I predict that golf course pairs will display 1) Decreases in nestling provisioning rates (reviewed in Frid and Dill, 2002, Bonier et al. 2007; Isaksson and Andersson, 2007; Ditchkoff et al. 2006), 2) Altered proportions of maternal vs paternal effort (Ditchkoff et al. 2006), 3) Increases in time spent nest building and incubating (McCarty and Secord, 1999; Weston and Elgar, 2007; Wang et al. 2008; Borneman et al. 2016), 4) Decreases in nest attendance rates (reviewed in Frid and Dill, 2002; Borneman et al. 2016), 5) Decreases in measures of reproductive output (reviewed in Frid and Dill, 2002; Bonier et al. 2007; Borneman et al. 2016), and 6) Different relationships between ornamental plumages, parental behaviors, and reproductive indices within sites.
**Predictions within Sites**

Within sites, under the good-parent-ornament hypothesis (Hoelzer, 1989), more ornamented individuals (e.g. higher UV tail chroma and darker melanin breast pigment) are predicted to 1) Provision nestlings at higher rates (Siefferman et al. 2003), 2) Spend more time in the vicinity of the nest (e.g. increased attendance), 3) Complete nests faster, and 4) Experience increased reproductive success (Siefferman et al. 2003; Siefferman and Hill, 2005a; Grindstaff et al. 2012).

**Methods**

**Study Sites:**

*High Disturbance Location.* A golf course (Hattiesburg, MS) serves as the high disturbance size for examination. This site has significantly higher percent open habitat and water, box density is significantly higher, and there is significantly higher human traffic passing boxes (Chapter I). Land-use and grounds maintenance also differ between sites (Chapter I, Table 1). I monitored 45 boxes here.

*Low Disturbance.* Camp Shelby Joint Forces Training Center ([Camp Shelby] Hattiesburg, MS) serves as the low disturbance site. There is little to no chemical maintenance of the grounds, a significantly higher percent of roads and buildings, significantly higher motor vehicle traffic passing boxes, and box density is significantly lower (Chapter I). I monitored 120 boxes here.
Study Species

The eastern bluebird is a socially monogamous, sexually dimorphic, bi-parental passerine (for full detail, see Chapter I). Bluebirds prospect for territories and mates in February and March and can produce up to three broods (clutch size: 2-5 eggs). Females build nests and incubate eggs while males assist in incubation provisioning of females. On average, females take 6 days to build nests and incubate eggs for 14 days (range 11-17 days in southern latitudes) (Gowaty and Plissner, 1998).

Capture and Monitoring

Capture occurred from 0400-1200h using either mist nets or box traps. I measured standard morphology (mass, tarsus length, wing chord, tail length) and fitted birds with an USFWS identification band (permit #23479-C) and unique combination of color bands. Preliminary study at the golf course began in 2012, and sites received full season monitoring in 2013 (golf course) and 2014 (Camp Shelby) while both sites were monitored concurrently in 2015. I monitored boxes daily for nest initiation and every other day for egg laying. I recorded stages of nest completion and time to complete nest (initiation date subtracted from nest cup completion), length of incubation, clutch size, and nestling number production as measures of reproductive output.

Plumage Analysis

Plumage data used in within-site correlations are those presented in Chapter II; both breast and tail plumage samples were collected after initial
capture of the bird. Briefly, both plumage color types were calculated using S2000 spectrometers, each geared toward their respective visual and UV ranges (reviewed in Andersson and Prager, 2006). For breast plumage, mean brightness or the percentage of light reflected from the feather, was calculated. For tail plumage, UV chroma, or spectral purity, was calculated (see Chapter II for full detail). Measures of melanin plumage were taken and calculated at the University of Southern Mississippi (L.M. Gillespie) while UV plumage samples were sent to Dr. Lynn Siefferman (Appalachian State University) for reflectance measures and calculation.

**Feeding Rates**

For assessment of feeding the number of trips taken to the box by both sexes with food was recorded. Birds were observed for 1 hour periods between 0500-1300 hours. Golf course birds were observed for a preliminary season late 2012, a full season in 2013, and an early 2015 season; Camp Shelby birds were observed for a full season in 2014 and an early season in 2015. I recorded the number of humans, number of cars or golf carts, and the number of military or maintenance vehicles that passed the nest (Chapter I). Some individual pair members were never caught for banding and were discarded from analyses; there were also occurrences of single parent attempts at provisioning at each site resulting in uneven sample sizes.
Nest Attendance Rates

Methods for collection of nest attendance were adapted from Ligon et al. (2012) (with assistance from M. Harris, Appalachian State). I observed nests for 5 trips taken (both sexes) or 1 hour of observation (regardless of trip number if maximum of 5 was not reached). Flags marking 10 and 40m from the nest box were placed (all visible from observation locations). At each site, nests in which forest edge or tree cover prohibited view of birds within or past 40m were discarded from analyses presented. During observations, I recorded: A) Time each pair member spent within 10m and within 40m of the nest, B) Time each pair member took during each trip to and from box, and C) Time away from box for total observation period (minutes/second) for each pair member. Observations presented are for when birds were visible for the entirety of an observation period. All times were converted to seconds. Results presented here are for average time spent (in seconds) by pairs within 40M of each box. These values were achieved by averaging individual pair member (male and female) time spent within 40 M of the nest; male and female feed rates are often correlated and males and females are assumed be communicating regarding nest vigilance (L. Siefferman, pers. comm).

Statistics

Distributions of variables (feeding rates, reproductive indices) were not normal, so all data were log +1 transformed for use in all analyses. Variables were examined for differences in year and then raw data was standardized for
year (using z-scores) if necessary. Both ANOVA and independent samples t-test were used when variables were transformed and compared between sites. Within sites raw data and Spearman’s Correlations were used to examine relationships between ornamental plumages and variables. I used a stepwise, Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) to control for false discovery rates resulting from use of multiple comparisons. This procedure sequentially reduces the number of comparisons, and the false discovery rate was set to 10% (McDonald, 2014).

Results

Year

Within each of the two study sites, there were no significant effects of year on proportion of nestling feeds, average feeding rates of females, or number of nestlings produced (Table 20). There was a significant difference between years for male feeding rates at the golf course (Table 20, Figure 28) and these were standardized for year (using z-scores) for use in future analyses.

Table 20

Annual Variation in Nestling Feeding

<table>
<thead>
<tr>
<th>Variable</th>
<th>Year</th>
<th>N</th>
<th>$F_{(df)}$</th>
<th>p</th>
<th>Year</th>
<th>N</th>
<th>$F_{(df)}$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prop. Nestling Feed</td>
<td>2013</td>
<td>21</td>
<td>0.1(1,31)</td>
<td>0.76</td>
<td>2014</td>
<td>17</td>
<td>0.762(1,27)</td>
<td>0.20</td>
</tr>
<tr>
<td>Female Feed Rate</td>
<td>2013</td>
<td>27</td>
<td>1.37(1,39)</td>
<td>0.248</td>
<td>2014</td>
<td>18</td>
<td>0.233(1,27)</td>
<td>0.63</td>
</tr>
<tr>
<td>Male Feed Rate</td>
<td>2013</td>
<td>22</td>
<td>8.15(1,32)</td>
<td>0.008*</td>
<td>2014</td>
<td>17</td>
<td>3.12(1,30)</td>
<td>0.09</td>
</tr>
</tbody>
</table>
N=sample size; F(df) = test statistic for ANOVA; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparison.

*Figure 28. Annual Variation in Male Provisioning*

Raw data is used in the graph. The golf course is represented in white and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and circles are beyond the 10th and 90th percentiles.

*Feeding Rates*

Independent samples t-test’s demonstrated no significant site differences in male feeding rates when both study years were combined (2013-2015) (Table 21, Figure 29). Females provisioned more often at the golf course compared to Camp Shelby (Table 21, Figure 29). In 2015, independent samples t-test demonstrated significant site differences in female feeding rates, with golf course...
females again showing significantly higher feeding rates (Table 21). In 2015, there were no site differences in feeding rates for males or proportion of male feeds (Table 21). Nest attendance rates of pairs (only measured in 2015) show that pairs at the golf course spend significantly more time within 40M of the nest box compared to pairs at Camp Shelby pairs (Table 21, Figure 30).

Table 21

Parental Care: Nestling Provisioning

<table>
<thead>
<tr>
<th></th>
<th>Golf Course</th>
<th>Camp Shelby</th>
<th>Independent Samples t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>All Years Female Feed Rate</td>
<td>0.41</td>
<td>0.03</td>
<td>40</td>
</tr>
<tr>
<td>Male Feed Rate</td>
<td>0.09</td>
<td>0.97</td>
<td>33</td>
</tr>
<tr>
<td>Prop. Of Feeds</td>
<td>0.47</td>
<td>0.24</td>
<td>32</td>
</tr>
</tbody>
</table>

|                      | M        | SD     | N  | M        | SD     | N  | T   | df | p     |
| 2015 Only Female Feed Rate | 2.05   | 0.95   | 13 | 1.18    | 0.43   | 9  | 2.50 | 20 | 0.02* |
| Male Feed Rate       | 2.36    | 0.88   | 11 | 1.47    | 0.46   | 14 | 3.25 | 23 | 0.004* |
| Prop. Of Feeds       | 0.49    | 0.18   | 11 | 0.50    | 0.11   | 8  | -0.13 | 17 | 0.895 |
| Nest Attendance      | 2.88    | 0.21   | 9  | 2.25    | 0.26   | 8  | 5.46 | 15 | <0.0001* |

M=mean; SD= standard deviation; n=sample size; t = test statistic; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.
Figure 29. Nestling Provisioning Rates

Raw data is used in the graph. The golf course is represented in white and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and circles are beyond the 10th and 90th percentiles.
Figure 30. Average Nest Attendance

Raw data is used in the graph. The golf course is represented in white and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and circles are beyond the 10th and 90th percentiles. Data are average time spent by pairs (male and female averages added together) within 40 meters of nest box during nestling feeding.

Reproductive Indices between Sites

Independent samples t-test show that golf course birds build nests more slowly compared to Camp Shelby birds (Table 22, Figure 31). Females at the golf course pairs lay smaller clutches (Table 22). There are no significant differences between sites in time spent incubating eggs (Table 22, Figure 32) or nestling age at fledging, however Camp Shelby pairs fledge significantly more nestlings in 2015 (Table 22, Figure 33).
Table 22

Reproductive Indices

<table>
<thead>
<tr>
<th>2015</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
<th>Independent Samples t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>N</td>
</tr>
<tr>
<td>Time to Build Nest</td>
<td>1.32</td>
<td>0.28</td>
<td>25</td>
</tr>
<tr>
<td>Clutch Size</td>
<td>0.57</td>
<td>0.11</td>
<td>27</td>
</tr>
<tr>
<td>Time to Incubate</td>
<td>13.68</td>
<td>2.21</td>
<td>25</td>
</tr>
<tr>
<td>Nestling Time in Nest</td>
<td>1.22</td>
<td>0.07</td>
<td>20</td>
</tr>
<tr>
<td>Nestlings Produced</td>
<td>0.58</td>
<td>0.20</td>
<td>26</td>
</tr>
</tbody>
</table>

M=mean; SD= standard deviation; n=sample size; t = test statistic; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparison.

Figure 31. Time to Complete Nest
Raw data is used in the graph. The golf course is represented in white and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and circles are beyond the 10th and 90th percentiles.

Figure 32. Time to Complete Incubation

Raw data is used in the graph. The golf course is represented in white and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and circles are beyond the 10th and 90th percentiles.
Figure 33. Nestlings Fledged in 2015

Data presented are from first nesting attempt of the season. Raw data is used in the graph. The golf course is represented in white and Camp Shelby is represented in dark grey. The line in the box is the median, the boxes are the 25th and 75th percentiles, whiskers are the 10th and 90th percentiles, and circles are beyond the 10th and 90th percentiles.

Feeding Measures and Ornamentation within Sites

For both males and females, there are no significant correlations between feeding rates and plumage coloration (melanin pigmented and UV chroma) at either site for all study years (Table 23).

Ornamentation and Reproductive Indices within Sites

There were no significant correlations between reproductive indices (nest building time, incubation time, clutch size, nestling age at fledge) and plumage coloration (melanin pigment or UV chroma) for males or females within the golf course or Camp Shelby (Table 24).
### Table 23

**Feeding Measures and Ornamentation within Sites**

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV*Average Feeding Rate</td>
<td>$r_s$ 0.008</td>
<td>$P 0.965$ 33</td>
</tr>
<tr>
<td>Melanin*Average Feeding Rate</td>
<td>$r_s$ -0.140</td>
<td>$P 0.437$ 33</td>
</tr>
<tr>
<td><strong>2015</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV*Average Feeding Rate</td>
<td>$r_s$ -0.005</td>
<td>$P 0.985$ 11</td>
</tr>
<tr>
<td>Melanin*Average Feeding Rate</td>
<td>$r_s$ 0.077</td>
<td>$P 0.821$ 11</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All Years</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV*Average Feeding Rate</td>
<td>$r_s$ -0.234</td>
<td>$P 0.146$ 40</td>
</tr>
<tr>
<td>Melanin*Average Feeding Rate</td>
<td>$r_s$ 0.180</td>
<td>$P 0.266$ 40</td>
</tr>
<tr>
<td><strong>2015</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV*Average Feeding Rate</td>
<td>$r_s$ -0.116</td>
<td>$P 0.721$ 12</td>
</tr>
<tr>
<td>Melanin*Average Feeding Rate</td>
<td>$r_s$ 0.088</td>
<td>$P 0.787$ 12</td>
</tr>
</tbody>
</table>

Raw data is used in this analysis; $r_s =$ Spearman’s rho, correlation coefficient; N= sample size; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.
Table 24

Reproductive Indices and Ornamentation within Sites

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs</td>
<td>p</td>
</tr>
<tr>
<td>Female UV*Clutch Size</td>
<td>0.078</td>
<td>0.744</td>
</tr>
<tr>
<td>Female UV*Incubation</td>
<td>-0.171</td>
<td>0.497</td>
</tr>
<tr>
<td>Female UV*Nestling Time in Nest</td>
<td>0.311</td>
<td>0.259</td>
</tr>
<tr>
<td>Female UV*Time to Build Nest</td>
<td>0.070</td>
<td>0.771</td>
</tr>
<tr>
<td>Female Melanin*Clutch Size</td>
<td>0.026</td>
<td>0.911</td>
</tr>
<tr>
<td>Female Melanin*Incubation</td>
<td>-0.148</td>
<td>0.545</td>
</tr>
<tr>
<td>Female Melanin*Nestling Time in Nest</td>
<td>0.168</td>
<td>0.550</td>
</tr>
<tr>
<td>Female Melanin*Time to Build Nest</td>
<td>-0.231</td>
<td>0.327</td>
</tr>
<tr>
<td>Males UV*Clutch Size</td>
<td>-0.302</td>
<td>0.161</td>
</tr>
<tr>
<td>Males UV*Incubation</td>
<td>0.294</td>
<td>0.184</td>
</tr>
<tr>
<td>Males UV*Time Spent in Nest</td>
<td>-0.306</td>
<td>0.217</td>
</tr>
<tr>
<td>Males UV*Time to Build Nest</td>
<td>0.082</td>
<td>0.710</td>
</tr>
<tr>
<td>Males Melanin*Clutch Size</td>
<td>-0.352</td>
<td>0.118</td>
</tr>
</tbody>
</table>
Table 24 (continued).

<table>
<thead>
<tr>
<th>Spearman’s Correlation</th>
<th>Golf Course</th>
<th>Camp Shelby</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male Melanin*Incubation</td>
<td>$r_s = -0.130$, $p = 0.585$, $N = 20$</td>
<td>$r_s = -0.087$, $P = 0.758$, $N = 15$</td>
</tr>
<tr>
<td>Male Melanin*Time Spent in Nest</td>
<td>$r_s = 0.451$, $p = 0.08$, $N = 16$</td>
<td>$r_s = -0.530$, $P = 0.05$, $N = 14$</td>
</tr>
<tr>
<td>Male Melanin*Time to Build Nest</td>
<td>$r_s = -0.082$, $p = 0.739$, $N = 19$</td>
<td>$r_s = -0.035$, $P = 0.892$, $N = 18$</td>
</tr>
</tbody>
</table>

Raw data is used in this analysis; $r_s =$ Spearman's rho, correlation coefficient; $N =$ sample size; * = significant result after application of Benjamini-Hochberg step-wise correction for multiple comparisons.

Discussion

Summary

Results provide partial support for the hypothesis that habitats differing in level of human disturbance subtly impact parental care behaviors in eastern bluebirds. In 2015, golf course pairs took significantly longer to build nests (average of 12.5 days; species average is 5.9 days) and spend significantly more time within 40m of the nest box during offspring care compared to Camp Shelby pairs. Golf course females provisioned nestlings at significantly higher rates than Camp Shelby females in for all years and in 2015 only. There are no site differences in provisioning rates or proportion of feeding for all years or 2015 for males, however, golf course males experience significant annual variation in provisioning rates compared to Camp Shelby males. There were no site differences in incubation length; however, golf course incubation lengths were more variable compared to Camp Shelby. Golf course pairs produce significantly
smaller clutch sizes and fledge significantly fewer young while there were no differences in nestling age at fledging.

Under the risk disturbance hypothesis, predictions for reduced nestling provisioning and attendance were not supported (reviewed in Fried and Dill, 2002), as females at the golf course (more disturbed) displayed increased provisioning, golf course males showed no differences in provisioning, and golf course pairs displayed increased nest attendance in comparison to the less disturbed population (Camp Shelby). Predictions for reproductive output, however, were supported (reviewed in Frid and Dill, 2002) as golf course pairs produced smaller clutches and fledged fewer young.

Under the good-parent-ornament hypothesis (Hoelzer, 1989), predictions within sites were not supported, as feeding behavior was not significantly correlated with any measure of reproductive investment or plumage coloration in males or female at either site for all study years and in 2015 alone. Reproductive measures within sites were also not correlated with plumage ornamentation, which is surprising considering at the golf course, both sexes are significantly more ornamented (Chapter II).

**Nest Building**

In some songbird species, one sex contributes more to nest construction (Collias and Collias, 1984), and parental investment (e.g. provisioning) may relate to nest size or quality, leading to hypotheses that nest building behavior may be a sexually selected trait (Jose et al. 1998). Building behavior can be
influenced by urbanized habitat, with several species altering nest sizes with increasing urban gradient (Reale and Blair, 2005; Wang et al. 2008). Additionally, tree swallows (Tachycineta bicolor) (McCarty and Secord, 1999), American kestrels (Falco sparverius) (Fisher et al. 2006), and zebra finches (Taeniopygia guttata) (Rochester et al. 2008) exposed to exogenous, hormonally active substances display alterations in nest building behavior, and effects are sex-specific in the latter two species. Consistent with predictions, bluebird pairs take significantly longer to complete nests at the golf course, with Camp Shelby birds completing nests almost 2 weeks faster (Figure 31). As nest building is expected to take 6 days in bluebirds (Gowaty and Plissner, 1998), golf course birds appear to exhibit profound delays in nest construction.

Among bluebirds, females traditionally build nests independent of males (Gowaty and Plissner, 1998), however, golf course males are consistently observed assisting females with building (pers. obvs. L. M. Gillespie, J. M. Jawor). They gather nesting material, bringing it into the box themselves or present it to females (similar behavior observed with prey items during nestling provisioning). These behaviors were not quantified because, in other bi-parental species, males assist females with nest construction (reviewed in Cockburn, 2006), however, apparently this male-helping behavior is unusual for bluebirds (pers. comm. Lynn Siefferman). It may be that golf course females are slow to build nests and males may compensate by providing assistance to increase individual fitness.
Extended delays in nest completion could be explained by differential insect emergence or availability at the golf course compared to Camp Shelby, as birds have been shown to coordinate reproduction with seasonal peaks in prey abundance (Daan et al. 1988). Use of pesticides impacts both insect populations and communities and can result in altered reproductive timing and emergence (reviewed in Newman, 2015). Golf course birds may delay nest completion to coordinate with anticipated higher resource abundance later in the season. However, because attempts to collect insect quantification were unsuccessful at the golf course, this explanation is speculative.

Alternatively, delays in nest completion may be related to alterations in endocrine physiology of golf course females. Female dark eyed juncos (Junco hyemalis) given T implants display extreme lengths between nest initiation, completion, and first egg dates, and are less likely to develop brood patches (Clotfelter et al. 2004). Implants of T in female zebra finches can reduce clutch sizes (Rutkowska et al. 2005), and golf course females have higher T (in response to conspecific intrusions), delayed egg laying (Chapter IV), and produced smaller clutch sizes. It may be that elevated T in some golf course females is a consequence of breeding density; boxes are more densely located at the golf course compare to Camp Shelby. Female bluebirds are highly territorial (Gowaty and Plissner, 1998), therefore, golf course females likely experience higher frequencies of conspecific encounters. This may elicit chronic and acute spikes in T during nest building, and which may alter both physiology
and behavior of females preparing to breed, resulting in delays in nest building and reductions in clutch sizes. Bluebirds can alter yolk deposition in response to intrusion (Navara et al. 2006), thereby potentially exposing developing embryos to altered levels of androgens by shunting T from maternal circulation (reviewed in Ubuka and Bentley, 2011). Given that golf course females display higher T (Chapter IV), lay smaller clutches, and receive mate assistance with nest building, it may be that female physiology (e.g. high T) is driving slower nest building at the golf course site.

**Incubation**

Incubation behavior for songbirds is similar to nest building; sexes either work in concert, or females perform the majority of incubation behaviors; development of brood patches (loss of ventral region feathers) and incubation behavior are both hormonally and environmentally regulated (reviewed in Vleck and Vleck, 2011). I found no significant difference between sites in incubation length contrary to predictions, however, the length of incubation at the golf course is highly variable (Figure 32). For the species as a whole, average incubation length is 14 days with a range of 11-17 days in southern breeding ranges (Gowaty and Plissner, 1998). Golf course females incubate within a range of 10-21 days, while Camp Shelby female incubation range is 12-16 days. Human presence can cause birds to abandon incubation bouts and spend more time off the nest (Borneman et al. 2016; Weston and Elgar, 2007) and such responses increase energetic expenditures (Yalden and Yalden, 1990). Human
activity at the golf course does not change greatly in the few weeks between incubation and nestling stages, and boxes here experience significantly higher human traffic during nestling feeding (Chapter I), so human presence is likely a contributing factor to variability in incubation lengths at the golf course. Additionally, male incubation behavior is observed at the golf course, this is likely unusual for this species as, to date, incubation has been described as a female-specific behavior in bluebirds (Gowaty and Plisser, 1998; Lynn Siefferman, pers. comm.).

**Reproductive Measures**

Females at the golf course produce significantly smaller clutches and pairs fledge fewer young compared to birds at Camp Shelby (data sampled in 2015). There are no significant differences in nestling age at fledging between sites. That fewer young fledge per clutch at the golf course is not surprising as it is a reflection of differences in clutch size. Previous research on bluebirds breeding on golf courses show variable results. A North Carolina population shows delays in egg laying and smaller clutch sizes at golf courses (Stanback and Seifert, 2005), a Virginia population, however, shows increases in egg and nestling production but lower nest survival at golf courses (LeClerc et al. 2005). In another VA population, reproductive output (e.g brood succes, nestling success, and nest prodcutivity) is higher at golf course sites (Cornell et al. 2011). Additionally, noise pollution can alter self-maintenance behavior and song in bluebirds (Kight and Swaddle, 2007; Kight and Swaddle, 2015). Taken together,
these results show large geographic variability in bluebird breeding success on golf courses and differential responses to anthropogenic disturbance.

In addition to differences in anthropogenic disturbance, habitat characteristics (percent water and open habitat) and nest box density differs between sites (Chapter I), as such, this may select for differences in abundance and species composition of predators seen at each site (Begon, 2005). Nests at the golf course were often depredated by fox squirrels (*Sciurus niger*) and argentine ants (*Linepithema humile*) while Camp Shelby nests were more often predated by snakes (L.M Gillespie, unpublished data). Differences in predator type or density within the golf course may select for smaller clutches, especially given that often at least 1 egg within a clutch fails to hatch on average (pers. obvs. L.M. Gillespie). Constraining clutch sizes this way would allow golf course birds to maximize reproductive productivity, as energetically, it may take less investment to provision a smaller brood. This may also help explain increased female feeding rates per chick at the golf course site.

*Nestling Provisioning*

In bi-parental species, contribution of both pair members is essential to nestling survival and adult fitness (reviewed in Cockburn, 2006). After hatching, nestlings require parental brooding until thermoregulation is possible, and similar to incubation, both brooding and nestling provisioning are associated with PRL in many avian species (reviewed in Vleck and Vleck, 2011). Nestling provisioning in both sexes can be negatively influenced by high levels of exogenous T (reviewed
in Ketterson et al. 2009; reviewed in Cain and Ketterson, 2012) and environmental stress (reviewed in Vleck and Vleck, 2011). My data demonstrate that, contrary to predictions made under the risk disturbance hypothesis (reviewed in Frid and Dill, 2002), females at the golf course provision nestlings at significantly higher rates in all study years and in 2015 than Camp Shelby females while there are no site differences for males.

Results for females at the golf course could simply result from smaller clutch sizes leading to smaller broods and increased provisioning rates. However, as feeding trips taken by females to the nest (with food, irrespective of nestling number) do not differ between sites as provisioning rates do, it is apparent that brood size may influence provisioning rates, although further investigation is required to determine how. It is surprising that golf course females provision at higher rates, given the obvious distress displayed by birds when human activity was especially high. During golf tournaments, birds would often cease provisioning for entire observation periods, consistent with other species responses to human disturbance (Clotfelter et al. 2004).

Insect quantification was unsuccessful at the golf course, however, golf course parents were observed foraging within 5-40+ m of nests and bringing prey items to the nest. Due to targeted elimination of pests resulting in different insect communities’ composition between sites (reviewed in Newman, 2015), golf course females may take more trips with lower quality prey items. Additionally, given constraints in clutch size (due to either high T or differences in predation
pressures), higher feeding rates likely allow females to fledge more young than possible if they laid larger clutches, as they can invest more time and energy later in the reproductive attempt. Golf course results are consistent, however, with another cavity nesting species, the great tit (*Parus major*) in which males in urban environments feed nestlings twice as often as those in rural environments (Isaksson and Andersson, 2007). Behavior alterations in response to human or environmental disturbances may be sex specific (Sih and Bell, 2004), as the sexes may react differently to disturbance events in general and when they provision nestlings.

There was significant annual variation in male provisioning rates at the golf course only (Figure 1). Potentially, because males are provisioning smaller clutches at the golf course, they invest energetically less overall, and therefore this trait may be more flexible with annual fluctuations in environmental factors. Additionally, male annual variation in feeding may be explained by female feeding rates, as golf course females feed at consistently higher rates annually (Table 1, Figure 2); males may adjust their feeding rate based on their mate’s increased investment.

*Nest Attendance*

General nest attendance is important for predator vigilance, mate and nestling guarding, and gauging needs of offspring; higher attendance is associated with increases in reproductive success in many species (reviewed in Lima, 2009; Ghalambor et al. 2013; Rastogi et al. 2006). Predictions for reduced
attendance at the golf course made under the risk disturbance hypothesis (reviewed in Frid and Dill, 2002) were not supported. For both sites surveyed in 2015, golf course pairs spend significantly more time within 40 meters of the nest box during the nestling phase compared to Camp Shelby pairs.

Prey abundance - although not measured- may explain these results. Birds breeding in habitat with poor food abundance often exhaust sources nearby quickly and must travel farther from the nest to forage and this reduces provisioning rates (Tremblay et al. 2005). Fire ants are also insectivores (reviewed in Vinson, 1997) and likely compete with bluebirds for provisioning resources. Bluebirds nesting in areas with experimentally reduced fire ant abundance forage closer to boxes, with the majority of foraging attempts within 50 meters of resident box, and these pairs provision at higher rates than those without ant reductions (Ligon et al. 2012). Potentially, golf course pairs are foraging closer to boxes, locating a more abundant (yet possibly lower quality) prey source not available to birds at Camp Shelby given increased fire ant density near nest boxes.

Fire ant abundance was quantified and for 2015 collections, all Camp Shelby samples consist of introduced, invasive fire ants (*Solenopsis invicta*), while all golf course samples consist of introduced, invasive argentine ants (L.M. Gillespie, unpublished data). Fire ants and argentine ants both impact avian and arthropod communities in their environment (Suarez et al. 2005; Ligon and Hill, 2010b; Estany-Tigerström et al. 2010). In terms of arthropods, argentine ants
specifically eliminate other native species of ants, impacting important caterpillar species (Estany-Tigerström et al. 2010). The complete lack of fire ants at the golf course paired with argentine ant presence may alter prey diversity or abundance available to golf course living bluebirds. This may give golf course birds access to more abundant (yet lower quality) prey items closer to the box than Camp Shelby pairs have access to. This may explain increased attendance of pairs and higher provisioning rates of golf course females.

Relationships to Ornamentation

Avian ornamental plumages may indicate quality of parental care behaviors or body condition in males or females under the good-parent-ornament hypothesis (Hoelzer, 1989; reviewed in Griffith and Pryke, 2006; reviewed in Hill, 2006). Results observed here did not support this hypothesis. Within sites, nestling provisioning did not correlate with melanin pigmented breast color or UV tail chroma in either sex, nor did reproductive indices. This is surprising as more ornamented males and females provision offspring more often and experience increased reproductive success in an AL bluebird population (Siefferman and Hill, 2003; Siefferman et al. 2005). The extent to which plumage color signals parental investment may vary with latitude. Indeed, in barn swallows and common yellowthroats (Geothlypis trichas), the extent to which sexual selection acts on ornamental plumage varies with geographic location (Scordato and Safran, 2014; Whittingham et al. 2015).

Conclusions
Results demonstrate partial support for the hypothesis that habitats differing in level of human disturbance subtly effect behavior and physiology in eastern bluebirds. Predictions for decreases in golf course productivity (latency in nest completion, small clutch sizes and reductions in offspring produced) are supported. For nestling provisioning and attendance, results were opposite of predictions, with golf course females only feeding at higher rates (for all study years and in 2015 only) and mated pairs displaying increases in nest attentiveness in 2015. Predictions for differences in relationships between ornamentation, feeding, and reproductive indices between sites were not supported but are interesting, given that golf course birds are significantly more ornamented than Camp Shelby birds (Chapter II).

Interesting differences exist for parental care behavior and productivity between sites. These may be driven by female physiology (e.g. higher T) or environmental and ecological differences (e.g. differential predation pressure, differences in composition of arthropod communities, increased human activity), but more than likely, a combination of all factors contributes to differences observed between sites. Site differences in disturbance of this nature, and, how both individuals and populations respond, requires further investigation.
APPENDIX A - IACUC Approval Letter

The University of Southern Mississippi
Institutional Animal Care and Use Committee
118 College Drive, #5147
Hattiesburg, MS 39406-0001
Tel: 601.266.6820
Fax: 601.266.5509
www.usm.edu/pac/policies/animals

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the three year approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes (see attached) should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 12042601
PROJECT TITLE: Impacts of human activity and different types of habitat maintenance regimes on eastern bluebird behavior and physiology
PROPOSED PROJECT DATES: 04/30/2012-09/31/2015
PROJECT TYPE: New
PRINCIPAL INVESTIGATOR(S): Jodie Jawor
DEPARTMENT: Biological Sciences
FUNDING AGENCY/SPONSOR: Full Committee Review Approval
IACUC COMMITTEE ACTION: 9/31/2015
PROTOCOL EXPIRATION DATE:

Gordon Cannon, Ph.D.
IACUC Chair

Date 9/24/15
REFERENCES


Cain, K.E., Ketterson, E.D. 2012. Competitive females are successful females; phenotype, mechanism, and selection in a common songbird. *Behav. Ecol. and Sociobiol.* 66(2), 241-252.

CALIFORNIA DEPARTMENT OF PESTICIDE REGULATION PUBLIC REPORT. 2008: Active Ingredient: Sulfosulfuron (Tracking ID Number 207805, 207806).


