INDIVIDUAL VARIATION IN TESTOSTERONE LEVELS AND PARENTAL CARE IN EASTERN BLUEBIRDS (*SIALIA SIALIS*): INTERACTIONS WITH REPRODUCTIVE SUCCESS AND OFFSPRING TRAITS

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Submitted to the Faculty of the
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in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2016

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ACKNOWLEDGEMENTS

Over ten years ago, I decided to pursue a life of working with and studying birds, and this dissertation is, thus far, the high point of that pursuit. There are many people to whom I owe gratitude. Without their help and advocacy, I would not have been able to complete my dissertation. First, I would like to thank my advisor, Dr. Jen Grindstaff. I am deeply grateful for her guidance and support, and her willingness to discuss hormonal mechanisms, emailing other faculty members, how to succeed as a woman in science, and everything in between. I owe many thanks to my committee members. I appreciate Dr. Barney Luttbeg for sharing his expertise in behavioral ecology and offering guidance on my statistical analyses. Dr. Sarah DuRant has offered a great deal of time and effort in helping me prepare my dissertation and plan what I would like to do next, which I very much appreciate. I thank Dr. Tim O'Connell for challenging me to think outside the realm of my research, which has made me a better scientist overall. I must also thank Dr. Alex Ophir, who provided feedback early on when I was developing my project, and Dr. Jennifer Hernandez Gifford, who helped me deepen my understanding of endocrinology. Many thanks to Dr. Britt Heidinger and Jeff Kittilson at North Dakota State University for teaching me qPCR and for advising me while I was working in their labs.

There are many students and members of the Stillwater community, too numerous to name, who served on the Bluebird Field Crew. I would not have been able to complete

my research without the help of these hard-working people. I especially owe thanks to Lisa Barrett, Wesley Grissom, Meg Hennessey, Susan Gleason, Anne Sabol, and Erin Reynolds for helping me learn how to be an effective mentor, and for remaining positive and diligent throughout our intensive field and lab work.

I thank my present and past labmates, Madeleine Naylor, Kaati Schreier, Meelyn Pandit, Danielle Perryman, Ann Money, Matt Waselik, and Jay Burtka Gatlin. Their suggestions and encouragement have been most helpful.

My dissertation research could not have been completed without financial support from the following sources: the Bob and Julia Bollinger Field Research Award, the Oklahoma Ornithological Society Research Award, the OSU Robberson Summer Dissertation Fellowship, the New York State Bluebird Society Research Grant, the North American Bluebird Society Research Grant, and the National Science Foundation Doctoral Dissertation Improvement Grant (IOS 1501408).

My fellow graduate students have been a great source of support during my time at OSU. I would especially like to thank my closest friends, Madeleine Naylor, Kaati Schreier, Lynne Beaty, Ashley Knoch, and Ashley Love. They have done more for me than words can express and I am grateful beyond measure.

I thank my parents, Rekha and Ashok, and my big brother, Anand, for helping to keep me grounded (and for making me laugh when I needed it). I thank David Levering for his belief in me over the years. Finally, I thank my sweet dog, Quigley, for being my constant companion, always.

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

Name: MEDHAVI AMBARDAR

Date of Degree: JULY, 2016

Title of Study: INDIVIDUAL VARIATION IN TESTOSTERONE LEVELS AND PARENTAL CARE IN EASTERN BLUEBIRDS (*SIALIA SIALIS*): INTERACTIONS WITH REPRODUCTIVE SUCCESS AND OFFSPRING TRAITS

Major Field: ZOOLOGY

Abstract: Hormones are important regulators of behavior and fitness. We have learned much about the direct effects of hormones on behavior and reproductive success from studies that experimentally manipulate hormone levels. To complement these studies, we also need to assess natural individual variation in hormones at multiple time points in relation to behavior and fitness as this practice can help to inform us about how hormonal profiles evolve. Testosterone has been implicated in fitness-related traits, and is predicted to interact with important behaviors, such as parental care. The level of parental care provided to young is critical in shaping the rearing environment. I conducted research on eastern bluebirds (*Sialia sialis*) to explore individual variation in testosterone levels in relation to parental care and fitness, and performed an experiment to alter parental behavior and measured subsequent effects on offspring. In Chapter II of my dissertation, I reported the findings of a study in which I injected birds with gonadotropin-releasing hormone (GnRH) to stimulate testosterone secretion within parental and aggressive contexts. I measured testosterone levels before GnRH was injected (initial testosterone levels) and thirty minutes after GnRH was injected (GnRH-induced levels), as well as the difference between these values (testosterone production). None of these testosterone measurements were related to nest visit rates or aggressive response to an intruder. However, there was significant variation among individuals in initial testosterone levels. Individuals also differed in their responsiveness to GnRH. In Chapter III, I showed that reproductive success was not related to initial testosterone levels or GnRH-induced testosterone levels. In the study conducted for Chapter IV, I manipulated brood sizes of bluebirds to create enlarged and reduced broods, keeping some broods unmanipulated as controls. Surprisingly, adults raising enlarged broods compensated for the increased number of young. As a result, the nestlings did not incur many costs overall, but nestling feather coloration was impacted. My findings emphasize the importance of studying individual variation in hormone levels, and identifying situations in which parents would be more likely to incur costs than offspring.

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CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

Hormones play a central role in regulating behavioral and reproductive traits (Adkins-Regan 2005). For practical reasons, many studies on the relationships among hormones, behavior, and fitness have used "phenotypic engineering" to artificially manipulate hormone levels and then study the subsequent effects on traits of interest (Ketterson et al. 1996). For example, males with artificially elevated testosterone levels have been shown to increase expression of mate-attracting behavior (De Ridder et al. 2000), engage in more extra-pair copulations (Raouf et al. 1997; Reed et al. 2006), defend larger territories (Chandler et al. 1994), increase competition with other males (Marler and Moore 1988), and reduce their contribution to care of young (Van Roo 2004). These overall patterns support the Challenge Hypothesis, which predicts that high baseline testosterone levels facilitate territorial aggression and mating (Wingfield et al. 1990). It also predicts that high baseline testosterone levels suppress parental behaviors (Wingfield et al. 1990), which are critical in shaping the offspring rearing environment (Saino et al. 1997; Leonard et al. 2000; Siefferman and Hill 2007).

Manipulations like those described above have been integral in establishing direct links between testosterone and behavioral and reproductive traits.

A potential drawback of manipulating testosterone with implants is that the manipulation maintains elevated testosterone levels for long periods of time, but in wild populations, it is more likely that short-term elevations of testosterone levels occur. Manipulations also do not capture natural individual variation in testosterone levels in relation to behavior and fitness. Such measurements are needed to assess how natural selection might act on hormonal profiles (McGlothlin et al. 2010). It is particularly beneficial to measure natural hormone levels at multiple time points, and the importance of this practice has recently been emphasized because it may allow us to determine the degree of flexibility of endocrine systems (Williams 2008; Taff and Vitousek 2016). Repeated sampling can also be used to examine if individual repeatability in hormone levels across time or contexts is related to personality (Duckworth and Sockman 2012). For instance, it has been hypothesized that individual repeatability in testosterone levels across time drives the repeatability that is observed in behaviors that are often mediated by testosterone (Duckworth and Sockman 2012; Burtka and Grindstaff 2013; Burtka and Grindstaff 2015).

Gonadotropin-releasing hormone (GnRH) challenges are a way to assess transient increases in testosterone levels (Jawor et al. 2006; McGlothlin et al. 2007; DeVries et al. 2012), and can be conducted on an individual multiple times (Jawor et al. 2006). Administration of exogenous GnRH stimulates the hypothalamic-pituitary-gonadal (HPG) axis to produce testosterone at an individual's natural levels, allowing researchers to measure GnRH-induced testosterone levels, as well as the magnitude of increase in

testosterone above pre-GnRH testosterone levels. In male dark-eyed juncos (*Junco hyemalis*), GnRH-induced testosterone levels are repeatable within individuals across time (Jawor et al. 2006), and are positively related to territorial behavior (McGlothlin et al. 2007), fitness measurements (McGlothlin et al. 2010), and the magnitude of increase in testosterone levels in response to GnRH is related to a sexually selected plumage ornament (McGlothlin et al. 2008). Using GnRH challenges builds on previous findings by relating important traits, such as parental care or reproductive success, to testosterone levels that likely reflect HPG axis responsiveness, rather than artificially elevated testosterone levels.

OBJECTIVES

In the next three chapters of my dissertation, I addressed the following primary objectives:

Chapter II) a. Characterize variation and repeatability of testosterone levels within individuals.

b. Assess relationships between parental investment (e.g., provisioning rates and nest defense) and GnRH-induced testosterone levels and testosterone production.

Chapter III) a. Test for relationships between fitness measurements, and initial testosterone levels and GnRH-induced testosterone levels in males and females.

Chapter IV) a. Examine how traits that are thought to be mediated by testosterone, such as parental investment, are impacted by brood size.

b. Assess potential costs to offspring in relation to parental investment (e.g., provisioning rates) and, thus, rearing environment.

METHODOLOGICAL OVERVIEW

I monitored a wild population of eastern bluebirds (*Sialia sialis*), a socially monogamous and biparental songbird (Gowaty and Plissner 2015), over three breeding seasons (2012 through 2014) in and around Stillwater, Oklahoma. In 2012, there were 160 nest boxes across eight nest box "trails." In 2013, I installed a ninth trail with an additional 13 nest boxes. I monitored nest boxes at least twice per week and recorded lay date, clutch size, hatch date, hatching success, fledge date and fledging success.

I analyzed parental care by videotaping provisioning behavior made by the adults when nestlings were 5–7 days post-hatch. I recorded adults on two separate days for two hours each and later analyzed the recordings to determine the number of times male and female bluebirds visited the nest box. I analyzed aggressive behavior when nestlings were 7–9 days old using a simulated territorial intrusion. For simulated territorial intrusions, I presented adult bluebirds with a live, caged house sparrow (*Passer domesticus*), a common nest competitor (Grindstaff et al. 2012). I conducted a two minute trial in which I observed the number of times males and females hovered over the cage, landed on the cage, and attempted to attack the sparrow within the cage (Grindstaff et al. 2012). I used

these behaviors to calculate an aggression score for males and females (Duckworth 2006; Grindstaff et al. 2012).

In 2012, 2013, and 2014, I conducted GnRH challenges on either the male or female of a pair after one randomly chosen parental care observation. I attempted to capture the same bird after the simulated territorial intrusion for a second GnRH challenge. After capture, I first took a blood sample to measure initial (pre-GnRH) testosterone levels (Jawor et al. 2006; McGlothlin et al. 2007; DeVries et al. 2012). I then injected the bird with GnRH and waited 30 minutes to allow testosterone levels to peak (Jawor et al. 2006; McGlothlin et al. 2007; DeVries et al. 2012). I took a second blood sample to measure post-GnRH testosterone levels (Jawor et al. 2006; McGlothlin et al. 2007; DeVries et al. 2012). I also calculated the difference between GnRH-induced and initial testosterone levels (testosterone production).

In 2014, I conducted a brood size manipulation to alter adult behavior and measure subsequent effects on offspring. When nestlings were two days old, I moved 1-2 nestlings between nests to create enlarged and reduced broods. I left the brood sizes of some nests unmanipulated, but removed nestlings for a short period of time and returned them to their original nest. These nests served as control broods. I measured parental and aggressive behavior as above. I also analyzed nestling mass, growth rates, stress hormone levels, telomere lengths, and feather coloration in relation to brood size group.

To address the objectives for chapter II, I used a likelihood ratio test to test for individual variation in initial and GnRH-induced testosterone levels. I also tested for relationships between initial testosterone levels, GnRH-induced testosterone levels and

testosterone production, and provisioning behavior and aggressive behavior. To address the objective for chapter III, I tested for relationships between the three testosterone measurements and fitness correlates, including adult mass, adult return rates, clutch size, fledging success, and offspring mass at fledging. I addressed the objectives for chapter IV by determining how brood size group affected adult behavior as well as the nestling measurements collected in 2014.

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CHAPTER II

PRE-GNRH AND GNRH-INDUCED TESTOSTERONE LEVELS DO NOT VARY ACROSS BEHAVIORAL CONTEXTS: A ROLE FOR INDIVIDUAL VARIATION

INTRODUCTION

In general, behavior is expected to be flexible within an individual, but repeatability in behavior across situations or time persists in a number of taxa (Budaev et al. 1999; Dingemanse et al. 2002; Johnson and Sih 2005; Briffa et al. 2008; Burtka and Grindstaff 2013; Burtka and Grindstaff 2015). One mechanism that may lead to behavioral repeatability is consistency of hormonal responses to a given stimuli that underlie a particular behavior (i.e., hormonal repeatability). If hormones and behavior are linked, then behavioral repeatability can reflect hormonal constraint on behavioral expression (Duckworth and Sockman 2012). Many studies on hormonal mediation of behavior are conducted by measuring means of the population sampled, but the importance of studying hormone-behavior relationships at the individual level (i.e., "beyond the mean") is becoming increasingly apparent (Williams 2008; Hau and Goymann 2015). Repeated sampling of individuals to assess variation in hormone levels

over time or across contexts is a necessary step in determining if repeatability of hormone levels constrains behavior (Duckworth and Sockman 2012).

The hormone testosterone (T) is often linked with the expression of parental and aggressive behavior, but it is unclear whether testosterone constrains an individual's behavior. The relationships between behavior and T are predicted by the Challenge Hypothesis (Wingfield et al. 1990). According to the Challenge Hypothesis, high levels of T in male birds are expected to increase the likelihood of the expression of territorial aggression (Wingfield et al. 1990). Aggressive behavior can be incompatible with parental care if individuals spend more time defending territories, and as a consequence, allocate less time to the care of young. As a result, high levels of T become associated with reduced parental care (Wingfield et al. 1990). The Challenge Hypothesis also predicts seasonal variation in T levels such that T is expected to be higher earlier in the season when territories are being established than later in the season (Wingfield et al. 1990).

While the predictions of the Challenge Hypothesis apply primarily to baseline T levels, obtaining accurate measurements of baseline T levels in the field can be difficult. However, measuring induced levels of T might more accurately reflect individual variation in T production (Jawor et al. 2006). One way to measure induced T levels is with gonadotropin-releasing hormone (GnRH) challenges (Jawor et al. 2006; McGlothlin et al. 2007), which were shown to produce individually repeatable T levels in dark-eyed juncos (*Junco hyemalis*) (Jawor et al. 2006). In a GnRH challenge, exogenous GnRH is injected to test the responsiveness of the regulatory mechanism involved in T secretion, the hypothalamic-pituitary-gonadal (HPG) axis (Jawor et al. 2006; McGlothlin et al.

2007). Administration of GnRH stimulates the anterior pituitary to secrete luteinizing hormone, resulting in T release by the gonads. By comparing GnRH-induced T levels to initial (pre-GnRH) T levels, we can quantify individual T production capabilities. Importantly, T levels produced in response to GnRH are expected to reflect those that individuals would experience during a social conflict (Jawor et al. 2006; McGlothlin et al. 2007). Such levels might be more tightly linked to behavioral expression than baseline levels (Jawor et al. 2006). Using the Challenge Hypothesis as a foundation, we can extend the predictions to include how GnRH-induced T levels should covary with behavior.

The goal of this study was to assess individual variation in baseline and GnRH-induced T within different behavioral contexts, and how GnRH responsiveness relates to the expression of parental and aggressive behavior. I used a wild population of eastern bluebirds (*Sialia sialis*), a common, cavity-nesting songbird, to study individual variation in T, and interactions between T and behavior. Eastern bluebirds are socially monogamous and exhibit biparental care in feeding of the offspring (Gowaty and Plissner 2015). They readily nest in human-made nest boxes in addition to natural cavities (Gowaty and Plissner 2015) and are limited by available nest sites. As a result, they can experience aggressive competition for nest boxes with conspecifics or heterospecifics (Burtka and Grindstaff 2013; personal obs.). In our population, parental provisioning behavior and nest defense in response to a heterospecific intruder are repeatable traits (Burtka and Grindstaff 2013; Burtka and Grindstaff 2015).

Baseline androgen levels are not related to parental care or aggression in this population (Burtka et al., 2016). One potential explanation for this pattern is that GnRH-

induced levels of T or the change in T (difference between GnRH-induced and initial levels) might better predict behavior because these levels reflect responsiveness of the HPG axis. Thus, building on the Challenge Hypothesis, I predicted that parental behavior would be negatively correlated with individual GnRH-induced T, and change in T (hereafter, "T production"). I also predicted that aggressive behavior would be positively correlated with individual GnRH-induced T and T production. Further, I predicted that T levels would vary seasonally. I tested the relationships between parental and aggressive behaviors and initial T levels as in previous studies (Burtka et al., 2016)

I also sought to quantify individual variation in T levels. I predicted that individuals would vary consistently from one another in both initial and GnRH-induced T levels across behavioral contexts. I tested this prediction using a reaction norm approach in which I analyzed variation in individual intercepts, which represent initial T levels, and variation in individual slopes, which represent responsiveness to GnRH (*sensu* Lendvai et al. 2014). In addition, T levels might be repeatable within individuals. Repeatability of T levels might constrain behavioral expression (Duckworth and Sockman 2012) and lead to repeatable nest defense behavior (Burtka and Grindstaff 2013) and nestling provisioning (Burtka and Grindstaff 2015) in our bluebird population.

METHODS

Nest box monitoring

I conducted fieldwork during the breeding seasons (March – August) of 2012 and 2013. All monitoring, observations, and blood sampling took place at previously established bluebird nest box trails in and around Stillwater, Payne County, Oklahoma,

USA (36°7′18″N 97°4′7″W). In 2012, I conducted fieldwork at eight nest box trails with a total of 160 nest boxes that had been in place since 2009. In 2013, I included a ninth trail with an additional 13 boxes that were put in place in October 2012. During the breeding season, I monitored all nest boxes twice per week to track nesting progress. When I found a complete nest, I began to check the box daily to determine laying date. I also checked the boxes daily around the projected hatch date (date of clutch completion plus 13 days) and the projected fledge date (date of hatching plus 16 days).

Adult measurements

I captured all adult bluebirds in the nest box using a prop trap. Females were caught late in incubation or during nestling rearing. Males were caught when nestlings were at least 4 days post-hatch. At the time of capture, I measured the mass of each bird, then marked them with an aluminum U.S. Fish and Wildlife Service (USFWS) band, and a unique combination of three plastic color bands.

Parental care

I videotaped parental behavior of adult bluebirds in 2012 and 2013. Nest boxes were videotaped on two separate days between 0700 and 1200 when nestlings were 5–7 days post-hatch with a Sony HDR CX260 digital video camera on a tripod at least 10 m from the nest box. Total recording time was between two hours and fifteen minutes and two hours and thirty minutes. During subsequent video analysis, I recorded the latency to the first nest box visit by either the adult male or female. I then determined the number of times adult male and female bluebirds entered the nest box (presumably to feed the

nestlings), and how much total time male and female bluebirds spent inside the nest box (a proxy for nest attendance) for two hours after the initial sighting. To determine individual variation in T production, I conducted a GnRH challenge immediately following one randomly chosen recording session for each nest box.

Aggressive behavior

I conducted simulated territorial intrusions in 2012 and 2013 between 0700 and 1200 on all pairs when nestlings were 7–9 days post-hatch to determine variation in aggression. For the intrusions, I used a live male house sparrow (*Passer domesticus*), a common nest competitor, as the intruder. Burtka and Grindstaff (2013) demonstrated that male and female bluebirds in our population do respond aggressively toward house sparrows.

Detailed methods for the simulated territorial intrusions are described elsewhere (Grindstaff et al., 2012). Briefly, I placed a house sparrow into a covered, steel cage that I attached to the top of the focal bluebird pair's nest box. I retreated to a blind or natural vegetation at least 15 m away and remotely removed the cover from the cage when the bluebird pair was within 100 m of the nest box. I ran a two minute observation period during which I recorded the number of times male and female bluebirds hovered within 0.5 m of the cage, landed on the cage, and attempted to attack the sparrow within the cage (*sensu* Duckworth 2006; Burtka and Grindstaff, 2013). The responses of male and female bluebirds to the house sparrow were used to calculate a score for each sex on a scale from 1–6, with 6 being the most aggressive (*sensu* Duckworth 2006). I attempted to catch the

same bird on which I conducted GnRH challenges within a parental context, and conducted another GnRH challenge immediately following the intrusion

To ensure that the bluebirds were responding to the house sparrow rather than the cage, I also conducted an empty cage trial on a subset of nests (2012: N=12; 2013: N=8). The empty cage trials were identical to the intrusions except I did not put a sparrow into the cage. None of the bluebird pairs responded aggressively to the empty cage.

GnRH pilot study to determine dosage

During May 2012, I conducted a pilot study to test the efficacy of GnRH challenges and to determine the appropriate dose and sampling time point. I captured adult birds when nestlings were 9–12 days old. Each bird was kept in a small cage and transported to the OSU Zoology Field Building for blood sampling and GnRH challenges. I first randomly assigned each bird to one of two groups: 1) low dose, in which four birds (2 males, 2 females) received one injection of 1.25 μg GnRH (American Peptide Company, #54-8-24) dissolved in 50 μl phosphate buffered saline (PBS) in the right side of the pectoralis muscle; 2) high dose, in which five birds (2 males, 3 females) received an injection in *both* sides of the pectoralis muscle (dosage: 2.50 μg GnRH in 100 μl PBS). I first took a blood sample (~50 μl from the jugular vein) to estimate initial T. I then administered the GnRH injection(s). The bluebird was kept in a covered cage and offered food (mealworms, larvae of the beetle *Tenebrio mollitor*) and water *ad libitum*. Thirty minutes after administering GnRH, I collected a second 50 μl blood sample. I took additional 50 μl blood samples at one hour and two hours after administering GnRH. In total, I collected blood at four time points, allowing the bird to rest in the covered cage

between sampling. After the final blood collection, the bird was allowed 15-30 minutes of cage rest before being released at the nest site. Total holding time ranged from 165 to 216 minutes (mean ± SE = 186.20 ±5.44 mins). I monitored the bird after release for at least 15 minutes and attempted to re-sight the bird 1-2 days after GnRH challenges and blood sampling to ensure that the bird had not been negatively affected. All of the birds in the pilot study appeared to behave normally shortly after release, and all were re-sighted near their nest box in the days following GnRH challenges. None of these birds abandoned their nests.

Plasma samples from the pilot study were stored at -20° C until analysis by enzyme immunoassay (EIA) (Assay Designs, #901-065) to determine T concentrations. Following Wada et al. (2007), I optimized the kit for use with eastern bluebirds to determine the appropriate plasma dilution and concentration of steroid displacement reagent (SDR). Briefly, I stripped plasma using 1% charcoal and 0.1% dextran in water to remove endogenous hormones. I then spiked the plasma to ~500 pg/ml using the T standard from the assay kit. I ran samples (in triplicate) against a standard curve at plasma dilutions of 1:10, 1:20, and 1:30, each with 0, 1, 1.5, and 2% SDR. Based on the optimization, I determined that for subsequent assays, plasma should be diluted 1:30 with no SDR added. Samples were run in duplicate and compared to a standard curve made with five standards (2,000 pg/ml, 500 pg/ml, 125 pg/ml, 31.25 pg/ml, and 7.81 pg/ml) added in triplicate to the plate. Plates were read on a Biotek ELx808 microplate reader at 405 nm.

Blood sampling: GnRH challenges

To determine T production capabilities within parental and aggressive contexts, I conducted GnRH challenges following one parental care recording session, and again on the same bird after the territorial intrusion. In 2012, I conducted GnRH challenges on males only. I performed GnRH challenges on 10 males within a parental context, and on 6 of those males again within an aggressive context. In 2013, I conducted GnRH challenges on males and females. I performed GnRH challenges on 18 males and 19 females within a parental context, and on 11 of those males and 6 of those females again within an aggressive context. I randomly chose which sex would receive a GnRH challenge at each nest, and I attempted to perform GnRH challenges on this bird after both the parental care trial and territorial intrusion. I only conducted GnRH challenges on one adult at each nest (i.e., not on breeding pairs of females and males). For the GnRH challenges, I collected a blood sample (~100 µl) to quantify initial T. I then injected the bird with the appropriate dosage of GnRH (described above). I held the bird in a bag for 30 minutes to allow T levels to peak. Thirty minutes after the GnRH injection, I took a final blood sample to quantify T levels in response to GnRH (Fig. 1). Plasma samples were stored and analyzed as described above for the pilot study.

Statistical analyses

All statistical analyses were performed in R version 3.2.0 (http://www.r-project.org). T values were not normally distributed, so I natural log transformed them. Residuals of all models were checked to ensure that assumptions of normality and homoscedasticity were satisfied. I first used linear mixed models with natural log

transformed T as the response variable, behavioral context (parental or aggressive) and sample (initial or GnRH-induced) as predictors, and individual as a random effect to determine how birds responded to GnRH within parental and aggressive contexts. I ran separate analyses for males and females. These analyses allowed us to determine mean T level responses for males and females.

To examine variation among individuals in T concentrations across behavioral contexts, I determined the optimal random structure for a linear mixed effects model to quantify the relative importance of individual elevation (intercept) and slope (*sensu* Lendvai et al. 2014). Finding the optimal random structure allowed us to identify sources of individual variation in T. With this approach, I tested if variation among individuals in T levels is primarily due to variation in initial T levels (individual intercepts), or is primarily due to variation among individuals in responses to GnRH (individual slopes). To assess the optimal random effect structure, I created several models in which I altered the random effects. I first fitted a linear model that contained no random effects and was based on the general formula

$$Y_i = \beta_0 + \beta_1 X_i + \varepsilon_i \qquad (1)$$

where Yi is T for an individual i. The population level fixed intercept and fixed effect predictors are given by β_0 and $\beta_1 X_i$, respectively, and ε_i is the residual error. Modifying Equation 1, I included individual identity as a random effect to fit a mixed model:

$$Y_i = \beta_0 + \beta_1 X_i + b_i + \varepsilon_i \qquad (2)$$

where the random effect, b_i, allows for a random intercept model at the individual level (i.e., b_i is the individual-level deviation from the population intercept). Significant variation in the random intercept indicates that initial T levels vary among individuals. Equation 2 can be expanded to include random slopes at the individual level by fitting slope, in addition to intercept, as a random effect:

$$Y_i = \beta_0 + \beta_1 X_i + b_{0i} + b_{1i} X_i + \varepsilon_i \qquad (3)$$

where $b_{1i}X_i$ is the random slope term and provides the individual-level deviation from the population slope. Furthermore, with this model, the variance-covariance structure of the random effects can be altered, to create two different random intercept and slope models: one in which intercept and slope were correlated, and one in which intercept and slope were uncorrelated. If the slope and the intercept are correlated, then GnRH-induced T levels are correlated with initial T levels. If the slope and intercept are not correlated, then this means initial and GnRH-induced T levels are not related to one another, and selection could act independently on initial T levels and GnRH responsiveness.

Using the nlme package (Pinheiro et al. 2015), I fitted each model using restricted maximum likelihood (REML) and altered the random effect structure while keeping the fixed effect structure the same. The models included behavioral context (parental or aggressive) and sample (initial or GnRH-induced) as fixed effects, with log-transformed T as the response variable. The contribution of random effects to the model fit was tested using a likelihood ratio test (Zuur et al. 2009). The likelihood ratio test compares two

models (e.g., the model without a random effect and the random intercept model) and is calculated as –2 times the difference in log-likelihood of the two models. The test gives a χ^2 distributed test statistic with degrees of freedom being the difference between the parameters of the competing models.

To determine correlations between behavior and T, I created mixed models fitted with maximum likelihood (ML) with individual identity as a random effect to control for multiple sampling of individuals. I used nest box visits, time spent in the nest box, or aggression score as the predictor and initial T levels, GnRH-induced T levels, or T production as the response variable. To analyze changes in T levels across the breeding season, I used date in the season as the predictor and initial T levels, GnRH-induced T levels, or T production as the response variable.

I measured repeatability of T levels in birds that had multiple GnRH challenges performed on them. In this dataset, I included birds sampled in 2012 and 2013 as well as those sampled using the same methods in 2014 to increase my sample size for individuals with repeated measures. I had repeated samples for 20 males and 15 females. Seventeen males were sampled twice, two were sampled three times, and one male was sampled five times. Thirteen females were sampled twice, one was sampled three times, and one was sampled four times. I determined repeatability within individuals in initial T levels, GnRH-induced T levels, and T production. I used the R package rptR, which calculates repeatabilities from the within- and among-individual variance components of linear mixed models fitted with REML (Nakagawa and Schielzeth 2010). rptR also calculates standard errors and 95% confidence intervals through parametric bootstrapping (Nakagawa and Schielzeth 2010).

RESULTS

Determination of GnRH dosage

I found that both sexes were able to elevate T in response to high dose GnRH challenges. In other words, GnRH-induced T was higher than initial T for males and females given a high dosage (2.50 μg GnRH in 100 μl PBS) of GnRH. Testosterone levels were highest 30 minutes post-GnRH challenge for the high dosage group (mean natural log transformed T±SE=7.06±0.0.07 pg/ml). T was still elevated one hour post-GnRH challenge, but was not as high as it was at 30 minutes (mean natural log transformed one-hour T±SE=6.56±0.18 pg/ml). By two hours, post-GnRH challenge T levels were not significantly different from initial T levels (mean natural log transformed two-hour T±SE=5.93±0.32 pg/ml; mean natural log transformed initial T±SE=6.02±0.29 pg/ml; t_6=-1.25, P=0.26). The low dosage of GnRH was not sufficient to cause an increase in T production (repeated measures ANOVA, $F_{3,9}$=3.06, P=0.17, N=4). Based on these data, I used the high dosage of GnRH during subsequent field GnRH challenges of adult male and female bluebirds (DeVries et al. 2011).

Responsiveness to GnRH across behavioral contexts

Male untransformed initial testosterone levels ranged from 0.04–4.25 ng/ml (mean ± SE = 0.86 ± 0.13 ng/ml) and untransformed GnRH-induced testosterone levels ranged from 0.05–5.22 ng/ml (mean ± SE = 1.17 ± 0.17 ng/ml). Female untransformed initial testosterone levels ranged from 0.07–4.33 ng/ml (mean ± SE = 1.17 ± 0.22 ng/ml) and untransformed GnRH-induced testosterone levels ranged from 0.15–3.17 ng/ml (mean ± SE = 0.80 ± 0.09 ng/ml). Overall, male bluebirds within parental contexts

significantly increased T from the initial to the final blood sampling points in response to GnRH, indicating that they were, on average, physiologically capable of elevating T in response to exogenous GnRH ($F_{1,26}$=5.42, P=0.03; Fig. 2). Within aggressive contexts, males did not significantly increase T in response to GnRH ($F_{1,26}$=0.28, P=0.61; Fig. 2). Initial T levels (pre-GnRH), GnRH-induced T levels, and T production (difference between initial and GnRH-induced T) were not significantly different between parental and aggressive contexts (Initial T: $F_{1,26}$=1.16, P=0.30; GnRH-induced T: $F_{1,26}$=0.40, P=0.54; T production: $F_{1,26}$=1.51, P=0.23; Fig. 2). Female bluebirds did not increase T from initial to final blood samples in response to GnRH in either parental or aggressive contexts (parental: $F_{1,17}$=0.51, P=0.48; aggressive: $F_{1,17}$=0.97, P=0.38; Fig. 3). Initial T levels, GnRH-induced T levels, and T production were not significantly different between parental and aggressive contexts in females (Initial T: $F_{1,17}$=0.12 P=0.74; GnRH-induced T: $F_{1,17}$=1.35, P=0.31; T production: $F_{1,17}$=0.007, P=0.94; Fig. 3).

Individual variation in testosterone production

Because I noted that some individuals of both sexes did not increase, or even decreased T in response to GnRH (Fig. 4), I used the likelihood ratio test to determine if individual variation in the response to GnRH existed by fitting separate models for males and females. Based on the likelihood ratio test, the model with individual identity as a random intercept received the highest support in males (Table 1). For females, the optimal model was also that which contained a random intercept (Table 2). Model fit was not improved by including correlated or uncorrelated random slopes in addition to random intercepts. The strong support for the random intercepts model suggests that there

was significant variation in initial T levels among individuals. That model fit was not improved by including random slopes suggests that initial and GnRH-induced T levels were not correlated, meaning that I did not detect evidence that individuals differed in their responses to GnRH. Using the random intercepts structure, I examined the effects of predictor variables, behavioral context and initial or GnRH-induced sample, on T levels. There was no significant effect of behavioral context on T in either males or females (Table 3). In males, but not females, T levels were significantly higher in GnRH-induced samples than in initial samples (Table 3).

I assessed repeatability of birds sampled in 2012, 2013, and 2014, giving me a total of 20 males and 15 females. Initial T levels did not differ between parental or aggressive contexts (Fig. 2). The same was true for GnRH-induced T levels (Fig. 2). For each T measurement (initial, GnRH-induced, production), I grouped samples from parental and aggressive contexts together. Males were significantly repeatable in initial and GnRH-induced T levels, but not in T production (Table 4). Females were only significantly repeatable in GnRH-induced T levels, but not in initial T levels or T production (Table 4). Parental behavior was recorded on two separate days allowing us to calculate repeatability of parental care as well. I confirmed that, as previously found (e.g. Burtka and Grindstaff 2015), both males and females were significantly repeatable in nest box visits per nestling per 2 hours (Table 5). Males and females were also repeatable in time spent in the nest box per nestling per 2 hours in the current study (Table 5). I did not have enough repeated measurements to calculate repeatability of aggressive behavior in response to a house sparrow.

Because bluebirds varied in their responsiveness to GnRH, I tested the hypothesis that future reproduction is dependent on the response to GnRH (i.e., whether an individual increases or decreases T after GnRH challenge) because responsiveness to GnRH may indicate reproductive status. However, individuals with another breeding attempt after the brood during which the bluebirds were sampled were not more likely to exhibit an increase in T levels after GnRH challenge (binomial test: Males: Z=-0.80, P=0.42; Females: Z=1.22, P=0.22). This analysis does not take into account the magnitude of T production. When the same parameters (subsequent breeding attempt, season) were used to estimate the difference between GnRH-induced and initial T levels, a subsequent breeding attempt still had no effect ($F_{1,20}$=1.73, P=0.28). Alternatively, the likelihood that an individual is able to increase T could be related to body size such that larger individuals are more likely to increase T while smaller individuals are not. I also did not find support for this hypothesis. For males as well as females, the probability that an individual would increase or decrease T in response to GnRH was not significantly related to body mass (binomial test, males: Z=1.16, P=0.25; binomial test, females: Z=-0.49, P=0.63).

Relationships between testosterone levels and behavior

Males and females did not differ in provisioning rates (number of nest box visits per nestling per two hours; Paired Wilcoxon signed rank: P=0.76), but females had significantly greater nest attendance (time spent in the nest box per nestling per two hours) than males (Paired Wilcoxon signed rank: P<0.01). Contrary to my prediction, I did not find that parental behaviors for either sex were negatively correlated with T

levels. Male provisioning rates were not significantly related to initial T levels ($F_{1,24}$=2.01, P=0.25), GnRH-induced T levels ($F_{1,24}$=0.74, P=0.48), or T production ($F_{1,24}$=0.60, P=0.52). Similarly, male nest attendance was not significantly related to initial T levels ($F_{1,24}$=0.01, P=0.94), GnRH-induced T levels ($F_{1,24}$<0.01, P=0.99), or T production ($F_{1,24}$=0.07, P=0.82). As with males, there were no significant relationships in female bluebirds between provisioning rates and initial T levels ($F_{1,17}$=0.18, P=0.67), GnRH-induced T levels ($F_{1,17}$=0.39, P=0.55), or T production ($F_{1,17}$<0.01, P=0.96). Female nest attendance also was not related to initial T levels ($F_{1,17}$=0.26, P=0.62), GnRH-induced T levels ($F_{1,17}$<0.01, P=0.95), or T production ($F_{1,17}$=0.78, P=0.40).

Males and females did not differ in their aggression score (Paired Wilcoxon signed rank: P=0.56). Male aggression score was not significantly related to initial T levels ($F_{1,13}$=0.22, P=0.64), GnRH-induced T levels ($F_{1,13}$=0.72, P=0.41), or T production ($F_{1,13}$=0.19, P=0.67). Female aggression score was positively related to initial T levels ($F_{1,4}$=11.21, P=0.03) and GnRH-induced T levels ($F_{1,4}$=20.70, P=0.01), but was not significantly related to T production ($F_{1,4}$=0.03, P=0.87). It is important to note that these female results were from 6 individuals as I was unable to recapture all of the females for which I had parental context GnRH data and aggression score data. In addition, 4 of the 6 females sampled were very aggressive (aggression scores of 5 or 6), while the remaining two had very low levels of aggression (aggression scores of 1). None of the females sampled had moderate aggression scores (score of 3 or 4). Female T levels within aggressive contexts were not significantly different from T levels within parental contexts (initial: t_8=0.30, P=0.77; GnRH-induced: t_8=0.28, P=0.79).

Changes in testosterone across the breeding season

As the breeding season progressed, male initial and GnRH-induced T levels within parental contexts did not change (initial: $F_{1,27}$=0.02, P=0.89; GnRH-induced: $F_{1,27}$=1.36, P=0.31). However, T production within parental contexts had a non-significant tendency to decrease over the season ($F_{1,27}$=5.57, P=0.08). For females, I also found that T production within parental contexts decreased throughout the season ($F_{1,17}$=7.47, P=0.01), but this relationship appeared to be driven by a single individual. With the removal of this outlier, T production did not significantly change over the season ($F_{1,16}$=0.65, P=0.43). However, female initial T levels had a non-significant tendency to decrease over the season ($F_{1,16}$=3.43, P=0.08), and GnRH-induced T levels significantly decreased over the season ($F_{1,16}$=6.12, P=0.02). While T levels within parental contexts decreased over the season, there was no seasonal change in parental behavior. For both males and females, provisioning rates (males: $F_{1,27}$=0.73, P=0.46; females: $F_{1,17}$<0.01, P=0.97), and nest attendance (males: $F_{1,27}$=3.49, P=0.16; females: $F_{1,17}$=0.43, P=0.52) did not decrease over the season.

For T within aggressive contexts, there was no seasonal variation in male initial T levels ($F_{1,17}$=0.19, P=0.67), GnRH-induced T levels ($F_{1,17}$=0.80, P=0.78), or T production ($F_{1,17}$=0.02, P=0.90). Similarly, there was no seasonal variation in female initial T levels ($F_{1,10}$=0.80, P=0.43), GnRH-induced T levels ($F_{1,10}$=2.41, P=0.23), or T production ($F_{1,10}$=1.83, P=0.27) within aggressive contexts. Although T within aggressive contexts did not change over the season, aggression scores for both males and females decreased significantly over the breeding season (males: $F_{1,36}$=4.88, P=0.03; females: $F_{1,10}$=7.92, P<0.01).

DISCUSSION

At the population level, male eastern bluebirds are physiologically capable of responding to exogenous GnRH, but only in parental contexts and not within aggressive contexts (after territorial intrusion). Female bluebirds did not appear to respond to GnRH in either context. In addition, while on average males increased T in response to GnRH, at the individual level, some males increased or decreased T levels in response to GnRH. The Challenge Hypothesis predicts that T levels in male birds are positively associated with aggression and negatively associated with parental care, and T levels are highest early in the breeding season (Wingfield et al. 1990). Despite being physiologically capable of responding to GnRH, T levels (initial, GnRH-induced, production) were not associated with the expression of parental or aggressive behavior. Male bluebirds had the greatest response to GnRH early in the breeding season, and GnRH responsiveness tended to decrease as the season progressed. Thus, male bluebirds do not appear to follow all of the predictions of the Challenge Hypothesis.

Individual variation in testosterone production

As demonstrated by the likelihood ratio test, there was considerable individual variation in levels of initial T in both males and females. Model fit was not improved by inclusion of random slopes. However, due to a relatively small sample size, the statistical power to detect consistent individual differences in GnRH responses was limited. When I examined the qualitative responsiveness to GnRH at the individual level (Fig. 4), I found that some birds elevated T levels after GnRH challenges, as expected, but others actually decreased T levels. I explored two potential explanations for why some individuals

increase T levels and others do not. First, I considered that investing in a subsequent brood would increase the probability of individuals increasing T in response to GnRH challenge because a subsequent breeding attempt would require the ability to elevate T. I did not find support for this hypothesis as responsiveness to GnRH did not predict the likelihood of having another breeding attempt for either sex. In the multiple-brooded song sparrow (*Melospiza melodia*), baseline T levels were highest during initiation of the first brood, but dropped off and remained low for subsequent broods (Wingfield and Goldsmith 1990). Eastern bluebirds continue to feed fledged young while the next clutch is being initiated (Gowaty and Plissner 2015). Thus, surges of T to prepare for additional breeding opportunities might also interfere with post-fledging care. Second, I tested if body mass predicted the likelihood of increasing T in response to GnRH based on the assumption that elevating T levels requires energy, and larger birds would have an energetic advantage over smaller birds (Daan et al. 1990). Again, however, this hypothesis was not supported.

Repeatability of hormone levels is necessary for hormones to evolve because repeatability of traits could be due to some genetic basis, making such traits potentially heritable (Lessells and Boag 1987). Testing hormonal repeatability can also give us insight into the proximate mechanisms that underlie behavioral consistency (i.e., personality; Sih et al., 2004). To that end, relatively few studies have yet demonstrated links between hormonal and behavioral consistency. In *Egernia whitii*, a species of skink, males and females exhibit repeatability in both T and aggressive response to an intruder (While et al., 2010). In western bluebirds (*Sialia mexicana*) males are repeatable in aggressive behavior, but behavior was not predicted by androgen levels and androgen

levels were not repeatable (Duckworth and Sockman 2012). Male dark-eyed juncos had significant repeatability in GnRH-induced T levels (r=0.36), but not in initial T levels (r=0.11; Jawor et al. 2006). Male bluebirds in our population were significantly repeatable in initial T levels, and both male and female bluebirds were significantly repeatable in GnRH-induced T levels. As in previous work in our population, male and female bluebirds were also repeatable in nest box visits. Males and females were repeatable in time spent in the nest box as well, though this behavior was not repeatable in previous years (Burtka and Grindstaff 2015). However, even though I found repeatability in T levels and parental care, I did not observe a relationship between T levels and parental behavior. Thus, it is possible that another hormone is driving consistent behavior in our population. For example estradiol, a product of T aromatization, has been implicated in territorial aggression (Soma et al., 2000). Serotonin, a neurotransmitter, may also modulate aggressive behavior. Pharmacological manipulations using fluoxetine, a selective serotonin reuptake inhibitor, to increase serotonin levels in the brain have demonstrated that fluoxetine can reduce aggression in birds (Sperry et al. 2003) and fish (Clotfelter et al. 2007).

Within the HPG axis, individual variation could occur at numerous points, and variation in initial T can be indicative of this. Many studies have taken a top-down approach in assessing sources of T variation by examining the upstream components of the HPG axis, such as the role of the hypothalamus (Anjum et al. 2012). However, there is also evidence that the gonads play a critical role in repeatable variation in T levels in dark-eyed juncos (Rosvall et al. 2013; Bergeon Burns et al., 2014). In addition, cross-communication between the HPA and HPG axes can alter resource allocation to

reproduction and T secretion (Wingfield and Sapolsky 2003). These studies demonstrate that there are many potential sources of variation in T levels. Furthermore, variation in behavioral expression can arise due to sensitivity to T (Hews et al., 2012; Bergeon Burns et al. 2013). Differences in androgen receptor density in certain brain regions, including the hypothalamus and the ventromedial telencephalon, have been associated with differences in aggressive behavior (Hews et al., 2012; Bergeon Burns et al. 2013). In this study, I explored only a portion of the HPG axis with T levels as the endpoint, but quantifying variation at other parts of the HPG axis might reveal why some individuals responded to GnRH while others did not. Future directions in the study of individual variation in T levels and interactions with behavior will benefit by considering the complex nature of T production and response to GnRH, and how interactions with behavior and other physiological processes can be sources of variation in hormone levels.

Testosterone and behavior

Male bluebirds increased T on average in response to GnRH, at least within a parental context. Having this physiological capability might be beneficial for males depending on the situation. Experimental elevation of T caused male European starlings (*Sturnus vulgaris*) to spend more time singing, a behavior that is used to attract potential mates (de Ridder et al. 2000). However, while T-treated starlings increased their investment in courtship, they decreased their investment in parental care (de Ridder et al. 2000). This pattern illustrates the classic trade-off between mate attraction and parental care that T is thought to mediate.

I predicted a negative relationship between T levels and parental care. Surprisingly, in our population, none of the T levels I measured (initial, GnRH-induced, or T production) in male bluebirds were correlated with either parental or aggressive behaviors. Previous work on this population has similarly found that baseline total androgen levels are not related to parental care or aggression (Burtka et al., 2016). The Challenge Hypothesis predicts that seasonally-breeding male birds should have a positive relationship between T and courtship and aggression, and a negative relationship between T and parental care (Wingfield et al. 1990). Dark-eyed juncos, as predicted, have a negative relationship between GnRH-induced T and provisioning rates (McGlothlin et al. 2007). However, it is becoming evident that some species do not follow this pattern. For example, male northern cardinals (*Cardinalis cardinalis*), like bluebirds, are physiologically capable of elevating T in response to GnRH, but neither GnRH-induced T nor the difference in T between GnRH-induced and initial samples predicted parental behavior (DeVries and Jawor 2013). There have been other studies on birds that have not found support for a relationship between individual variation in T and parental behavior (reviewed in Lynn 2016). These studies accentuate the complex relationships between individual variation in T and expression of parental behavior.

Males of species that do not follow the predicted pattern between T and parental care might not do so because they are "behaviorally insensitive" to T (Lynn 2008). According to the "essential paternal care" hypothesis, behavioral insensitivity to T in males should occur in species in which male care is necessary for success of the nest (Lynn 2008). In these species, T is less likely to suppress male parental behavior because males may gain a greater fitness benefit from providing care to offspring than from

sexual behaviors or territorial aggression, traits which are typically associated with increased T (Lynn 2008; Lynn 2016). Male chestnut-collared longspurs (*Calcarius ornatus*) do not reduce parental care in response to elevated T, potentially because care provided by males is essential for offspring survival and thus, reproductive success (Lynn et al. 2002). I cannot explicitly conclude that care provided by male bluebirds is essential for survival of the young because I did not perform mate removal experiments, but I do know that males feed at rates similar to those of females (see above). Male contribution to parental care might be substantial enough in this species for behavioral insensitivity to T to have evolved.

Male aggression score also did not correlate with T levels in our population. Northern cardinals, as well as black redstarts (*Phoenicurus ochruros*) elevate T after being given GnRH, but do not elevate T in response to simulated territorial intrusions (DeVries et al. 2012; Apfelbeck and Goymann 2011). In our population, eastern bluebird T levels after a simulated territorial intrusion are not associated with aggressive behavior. For these trials, the initial T levels came from blood samples collected after the territorial intrusion. I was unable to determine if bluebirds increased T in response to an intruder because I did not sample birds before being presented with the house sparrow. However, behavioral insensitivity may be relevant here once again, at least in my study where I studied aggression within a nest defense, and therefore, parental investment, context.

In females, responsiveness to GnRH may be dependent upon the stage of nesting. I sampled females only during the nestling provisioning stage, but female dark-eyed juncos that were sampled across the nesting stage varied in their responsiveness to GnRH challenge (Jawor et al. 2007). Females increased T in response to GnRH only when

GnRH challenges occurred during egg development, but not during the other stages of nesting, including the nestling provisioning stage (Jawor et al. 2007). For female bluebirds, none of their T measurements were significantly correlated with parental behavior. Like males, the expression of female parental behavior may be sensitive to elevations in T levels. Female juncos with experimentally elevated T levels spent less time brooding their nestlings, and had reduced offspring survival compared to control females (O'Neal et al. 2008). Thus, the inability of female bluebirds to elevate T levels in response to GnRH during the nestling provisioning stage could be adaptive to minimize negative impacts of T on parental care and offspring survival.

Unlike parental behavior, female aggression score in bluebirds was positively associated with initial and GnRH-induced T levels. These results were from only 6 individuals whose aggression scores were either high or low, making it difficult to draw conclusions about patterns of T and aggression in females in the current study. However, a prior study in our population with a larger sample size for female bluebird androgen levels did not find a relationship between androgens and aggression score (Burtka et al. 2016). Further, while experimental treatment of birds with T causes females of some species to behave more aggressively (e.g., European starlings; Sandell 2007), it does not have the same effect in other species (e.g., dark-eyed juncos; O'Neal et al. 2008). The variation among species in modulation of female aggressive behavior by T may reflect differences in natural history.

Changes in testosterone production across the breeding season

The Challenge Hypothesis predicts seasonal fluctuations in T such that early in the breeding season, male T is elevated, which facilitates competition over territory establishment (Wingfield et al. 1990). Although not significant, male T production decreased over the season. This relationship may reflect increased selection pressure to be physiologically able to elevate T in the early part of the breeding season, relative to later in the season. Further, since high levels of T can inhibit molt (Schleussner et al. 1985), having lower T later in the season might be beneficial. Female bluebirds did not change in their responsiveness to GnRH over the season, similar to female dark-eyed juncos, which again suggests that GnRH responsiveness in females of some species might be strongest during certain stages of nesting cycle (e.g., during egg laying; Jawor et al. 2007).

Conclusion

Here, I demonstrate that T levels and parental care are repeatable within individual male and female eastern bluebirds, but T does not appear to be related to the expression of parental and aggressive behaviors in in this species. These findings confirm those of other studies and add to a growing body of literature suggesting that, in some species, T and may not modulate parental care and aggression. I also provide a novel test of individual variation in T levels which suggests that there is flexibility in initial T and T response to GnRH injection, regardless of context. Further study to identify sources of behavioral decoupling, as well as sources of individual variation in T will help to elucidate why some species might evolve behavioral insensitivity to T.

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Table 1. Test for individual differences in male T levels before and after GnRH challenge. The models tested had varying random effect structures. Inclusion of a random intercept in the model improved the predictive power, indicated by a likelihood ratio test to compare log likelihood (LL) values of candidate models.

Model	Model random structure	LL	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	-125.8	4			
2	Random intercept	-110.4	5	30.8	1 vs. 2	**<0.0001**
3	Correlated random intercept and slope	-109.5	7	1.86	2 vs. 3	0.50
4	Uncorrelated random intercept and slope	-110.4	6	-1.86	3 vs. 4	0.50

Table 2. Test for individual differences in female T levels before and after GnRH challenge. The models tested had varying random effect structures. Inclusion of a random intercept in the model improved the predictive power, indicated by a likelihood ratio test to compare log likelihood (LL) values of candidate models.

Model	Model random structure	LL	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	-67.4	4			
2	Random intercept	-49.2	5	36.5	1 vs. 2	**<0.0001**
3	Correlated random intercept and slope	-47.1	7	4.12	2 vs. 3	0.50
4	Uncorrelated random intercept and slope	-48.6	6	-3.10	3 vs. 4	0.50

Table 3. Effects of behavioral context and sample (initial or GnRH-induced) on natural log-transformed T levels (pg/ml). For males, T levels did not differ across behavioral contexts, but T levels were significantly different across samples, such that GnRH-induced T levels were higher than initial T levels. For females, T levels did not differ across behavioral contexts or across samples.

Sex	Predictor	Slope±SE	F	df	P
Male	Intercept	6.60±0.20	108.0	1, 54	**<0.0001**
	Behavioral context	0.11±0.17	0.50	1, 54	0.48
	Sample	-0.31±0.14	4.98	1, 54	**0.03**
Female	Intercept	6.53±0.20	105.2	1, 27	**<0.0001**
	Behavioral context	-0.12±0.17	0.52	1, 27	0.48
	Sample	0.11±0.11	0.99	1, 27	0.33

Table 4. Repeatability estimates for male and female T levels across parental and aggressive contexts. Males were significantly repeatable in initial and GnRH-induced T, but not in T production (difference between GnRH-induced and initial T levels). Females were significantly repeatable in GnRH-induced T, but not in initial T or T production.

Sex	T measurement	R (SE)	CI	P
Male	Initial T	0.51 (0.15)	[0.16, 0.74]	**0.005**
	GnRH-induced T	0.74 (0.10)	[0.50, 0.87]	**<0.001**
	T production	0.18 (0.15)	[0, 0.50]	0.15
Female	Initial T	0.39 (0.91)	[0, 0.71]	0.10
	GnRH-induced T	0.60 (0.17)	[0.18, 0.84]	**0.01**
	T production	0.05 (0.14)	[0, 0.49]	0.93

Table 5. Repeatability estimates for male and female parental behaviors. Both males and females were significantly repeatable in box visits per nestling per 2 hours and time spent in the box per nestling per 2 hours.

Sex	Parental behavior	R (SE)	CI	P
Male	Box visits per nestling	0.55 (0.14)	[0.24, 0.76]	**0.002**
	Time in box per nestling	0.45 (0.16)	[0.10, 0.70]	0.05
Female	Box visits per nestling	0.51 (0.14)	[0.18, 0.74]	**0.005**
	Time in box per nestling	0.40 (0.17)	[0.17, 0.67]	**0.006**

Figure 1. Schematic of GnRH challenges. When nestlings were 5–7 days old, I conducted parental care observations. After one randomly chosen parental care trial, I captured the male (2012) or either the male or female (2013) and performed a GnRH challenge. When nestlings were 7–9 days old, I conducted simulated territorial intrusions. Following the intrusion, I recaptured some of the same birds as before and again performed a GnRH challenge.

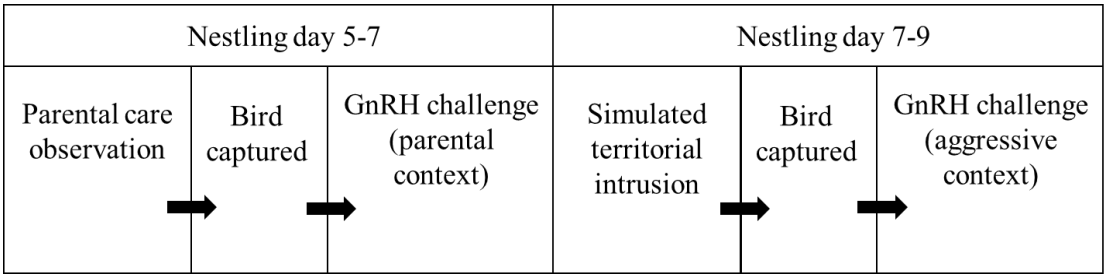


Figure 2. On average, male bluebirds increased T in response to GnRH within parental contexts, but not within aggressive contexts. Parental and aggressive T levels did not significantly differ from each other for initial and GnRH-induced samples.

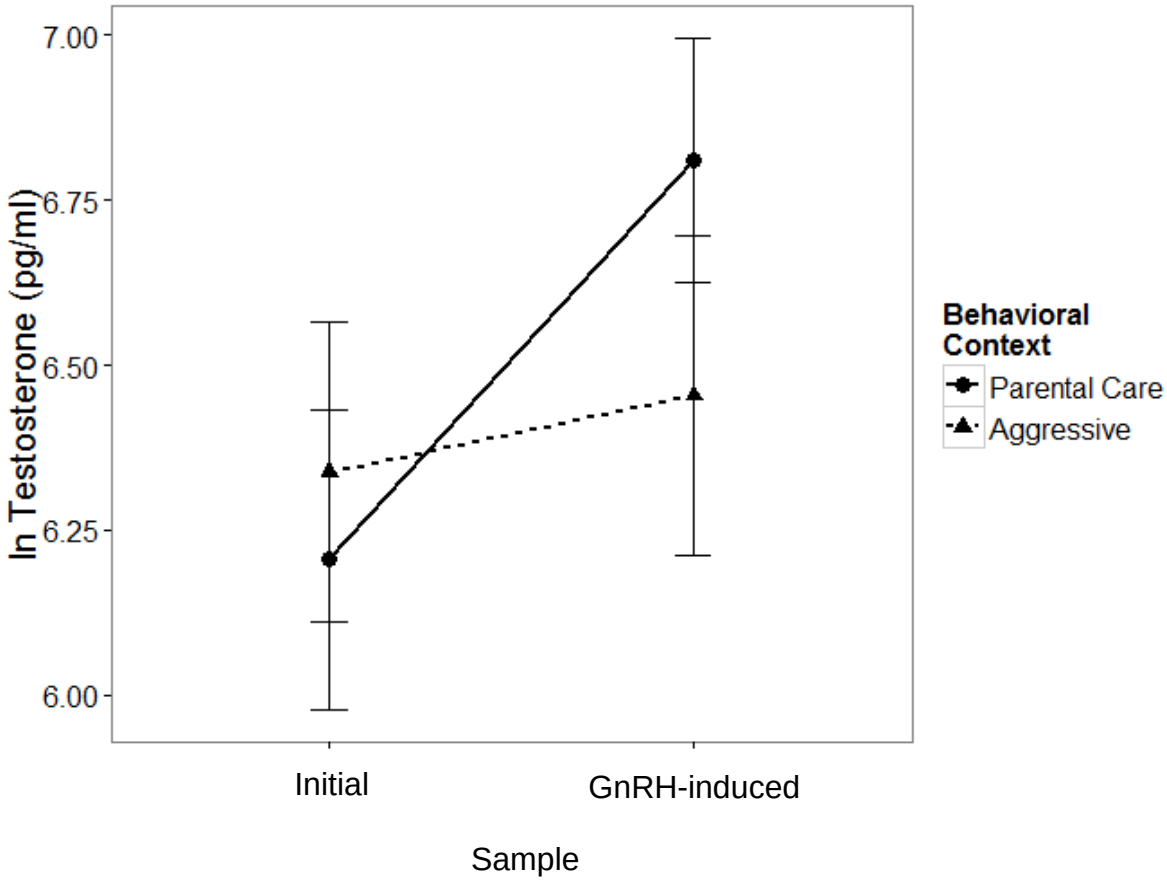


Figure 3. Female bluebirds did not significantly increase T in response to GnRH within either parental contexts or aggressive contexts. Female parental and aggressive T levels also did not differ from each other for initial and GnRH-induced samples.

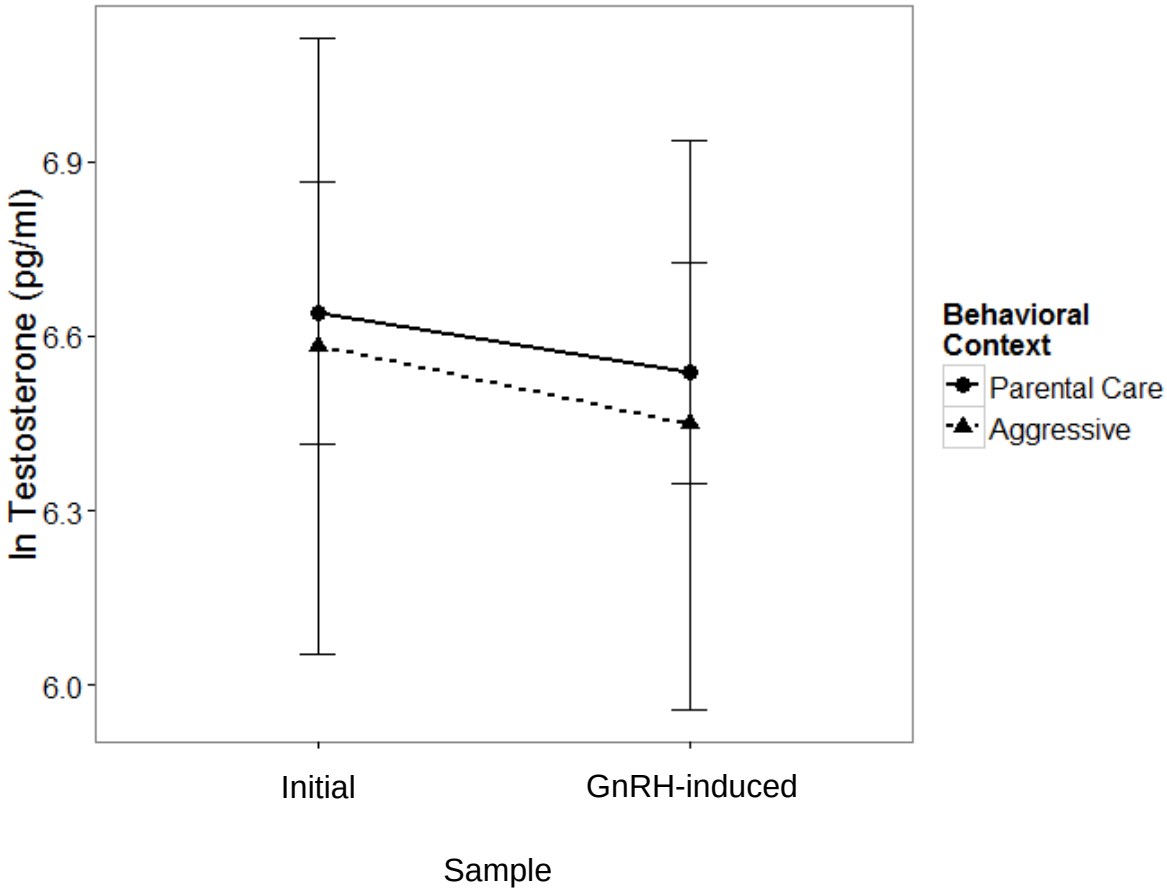
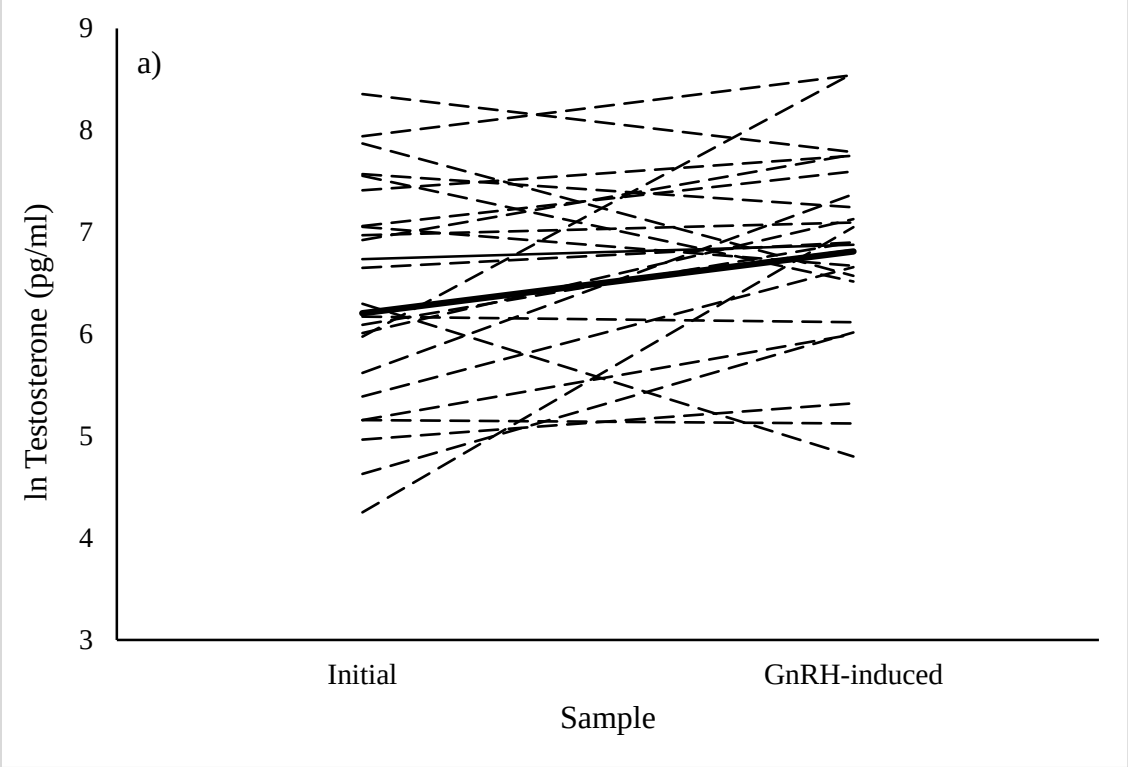
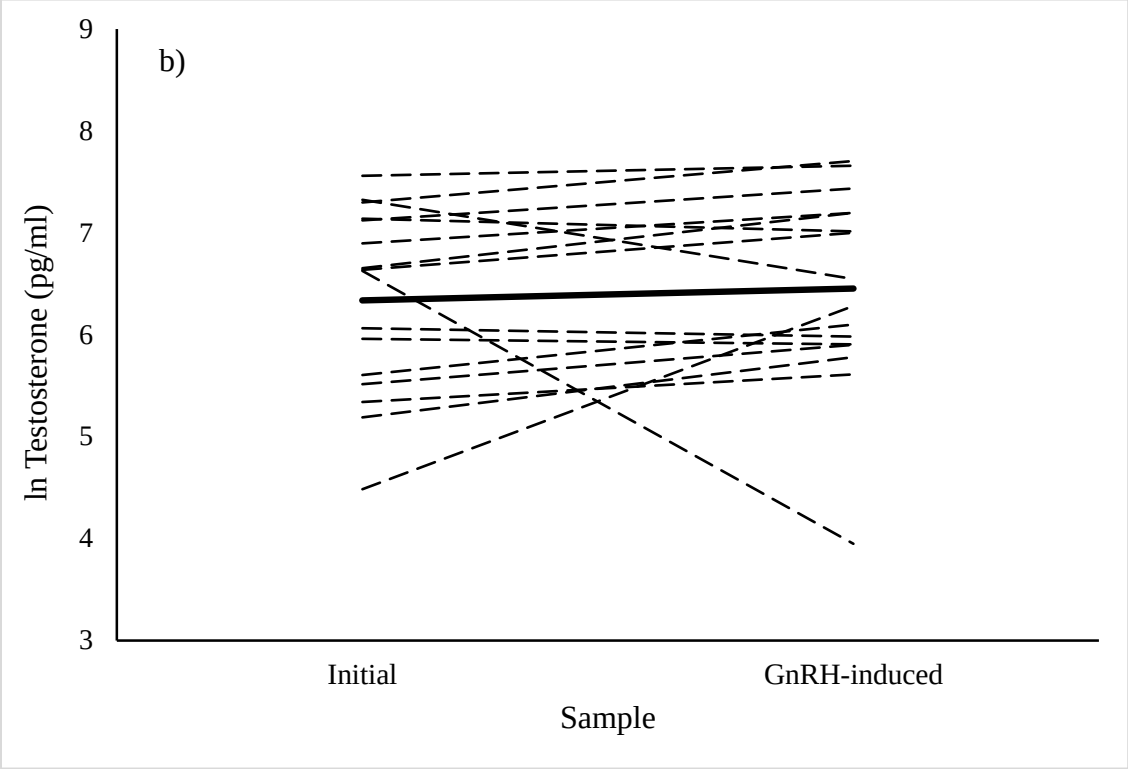


Figure 4. Individual T levels before (Initial) and after (GnRH-induced) GnRH challenges within a) parental contexts for males, b) aggressive contexts for males, c) parental contexts for females, and d) aggressive contexts for females. Thin dashed lines indicate individual responses, and thick solid black lines are population level means.

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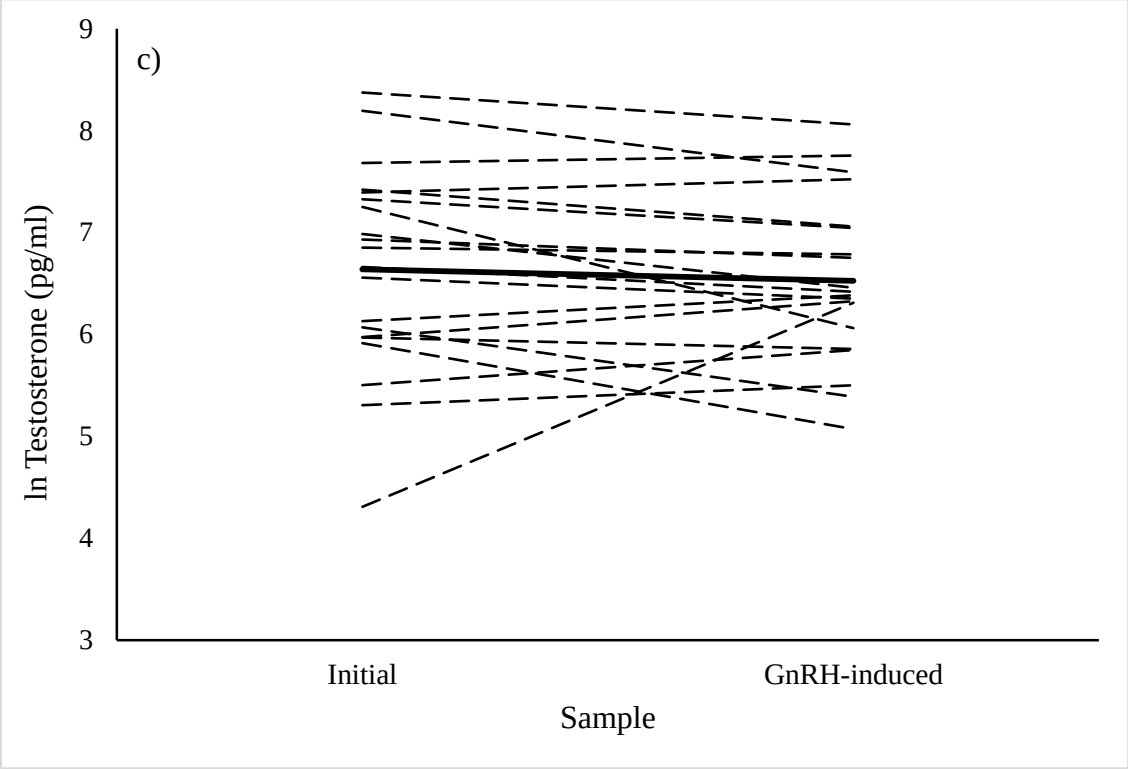
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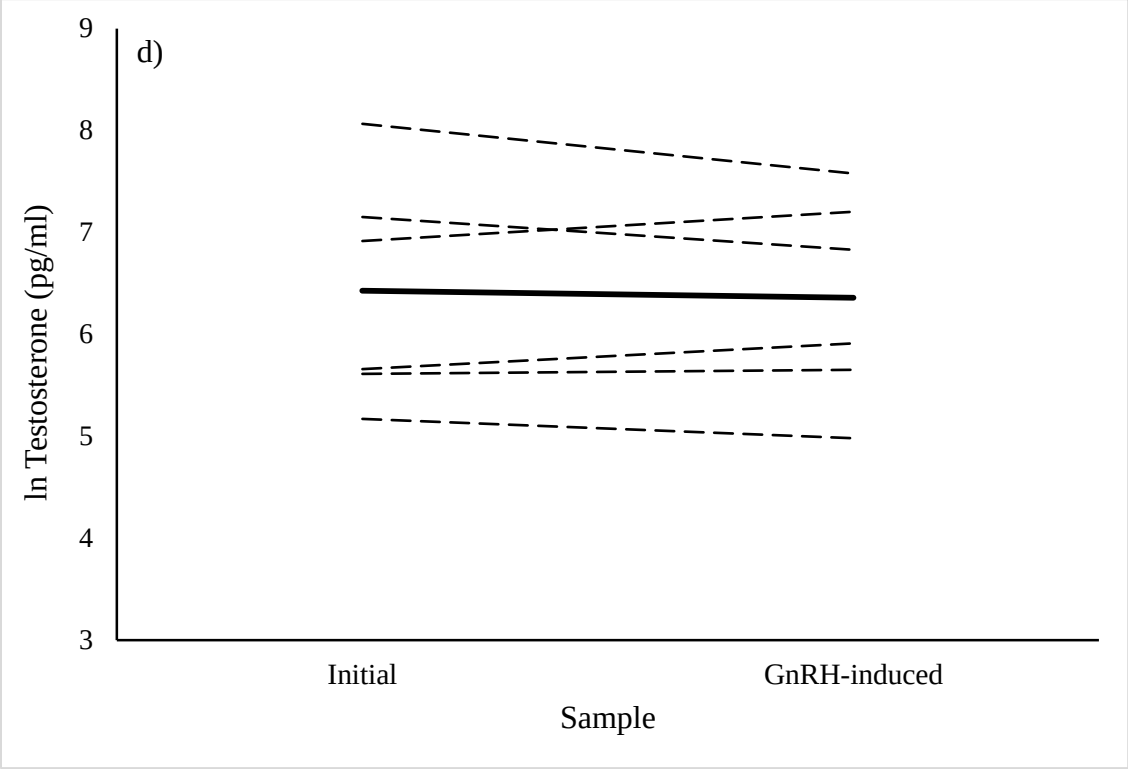
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CHAPTER III

GONADOTROPIN RELEASING-HORMONE-INDUCED TESTOSTERONE LEVELS PREDICT REPRODUCTIVE SUCCESS IN FEMALE AND MALE EASTERN BLUEBIRDS (*SIALIA SIALIS*)

INTRODUCTION

Hormones play a central role in the relationship between the phenotype and fitness because they mediate physiological processes and behaviors that are essential for reproduction. Thus, an understanding of the relationships between hormones and reproductive success may provide an avenue for understanding the evolution of hormonal profiles (Ketterson et al. 2005; McGlothlin et al. 2010; Ouyang et al. 2011). For logistical reasons, many studies on the relationships between hormones and reproduction have used experimental manipulations of hormones. However, measuring individual variation in hormone secretion is needed to assess how selection might act on hormone levels. This approach has been successfully used to study selection on testosterone levels in free-living populations (McGlothlin et al. 2010; Edwards et al. 2015; Smith et al. 2015).

Individual variation in testosterone has been linked to reproductive success in males. In black rhinoceros (*Diceros bicornis*), males that had recently sired offspring had higher fecal testosterone metabolite concentrations than males that had not recently sired young (Edwards et al. 2015). Furthermore, male rhinoceros with higher testosterone metabolite concentrations also sired more young per year (Edwards et al. 2015). Similarly, male copperhead snakes (*Agkistrodon contortrix*) with higher testosterone levels copulate more frequently than males with lower testosterone levels (Smith et al. 2015). In many birds, elevated male testosterone levels have been linked to increased expression of mate-attracting behavior (De Ridder et al. 2000), higher fitness from gains due to extra-pair copulations (Raouf et al. 1997; Reed et al. 2006), larger home ranges (Chandler et al. 1994), and greater territorial behavior (Wingfield et al. 1990; Alatalo et al. 1996).

Several costs have also been associated with high testosterone levels. Testosterone may reduce survival (Marler and Moore 1988; Nolan et al. 1992; Reed et al. 2006), due, in some cases to increased male-male competition (Marler and Moore 1988). There is also an expected trade-off between survival and reproduction that is thought to be mediated by testosterone. Testosterone-treated male dark-eyed juncos (*Junco hyemalis*) had reduced survival, but compensated by siring more young (Reed et al. 2006). Suppressed parental care in response to high testosterone levels may also lower fledging success in some birds (Hegner and Wingfield 1987). Together, these results suggest that, at least in males, testosterone might be closely tied to fitness via its effects on behavior, survival, and reproduction.

Compared to males, relatively little is known about the relationship between testosterone and fitness in females. To fully understand how hormonal profiles may evolve within a given species, it is important to understand how hormones are related to fitness in both sexes. It has been hypothesized that female testosterone levels may be a result of a correlated response to selection on male testosterone levels (Clotfelter et al. 2004; Ketterson et al. 2005). Based on this hypothesis, we would expect that female testosterone levels would be significantly correlated with male testosterone levels (Ketterson et al. 2005). Such a pattern has been found in some species, but not in others (Ketterson et al. 2005). Alternatively, female testosterone levels may arise not because of a correlated response to male evolution, but because of direct selection on female testosterone levels (Ketterson et al. 2005). We would then expect female testosterone levels to be directly related to female fitness (Ketterson et al. 2005). Experimental elevation of female testosterone levels in dark-eyed juncos negatively impacts reproductive success (O'Neal et al. 2008; Gerlach et al. 2013), and reduces expression of some parental behaviors (O'Neal et al. 2008). However, Veiga and Polo (2008) found that testosterone treated female spotless starlings (*Sturnus unicolor*) did not differ from control females in total reproductive output. Relationships between female reproductive success and testosterone deserve more study in order to identify common patterns across species

Many studies on both sexes, including some discussed here, have used "phenotypic engineering," to artificially alter testosterone levels and measure subsequent responses. Such studies are necessary to identify causal links between hormones and phenotypes. One drawback of these types of studies, however, is that they do not capture

natural variation in hormone levels that is present at the individual level. In addition, manipulated testosterone levels remain elevated for fairly long periods of time, but short-term elevations of hormone levels are probably more realistic in wild populations.

Gonadotropin-releasing hormone (GnRH) challenges provide a simple way to measure variation among individuals in short-term elevations of testosterone (Jawor et al. 2006; McGlothlin et al. 2007; DeVries et al. 2012). Natural secretion of GnRH from the hypothalamus initiates the release of luteinizing hormone and follicle stimulating hormone from the pituitary, resulting in production of testosterone from the gonads. This cascade of events in the hypothalamic-pituitary-gonadal (HPG) axis is also activated in response to social stimuli. In a GnRH challenge, we artificially stimulate the HPG axis by giving an individual an exogenous dose of GnRH. We can then measure how an individual's testosterone changes from baseline to GnRH-induced levels. In male dark-eyed juncos, GnRH-induced testosterone levels vary among individuals and are repeatable (Jawor et al. 2006), and are positively correlated with natural increases in testosterone levels that arise during male-male competition (McGlothlin et al. 2008).

Individual levels of GnRH-induced testosterone levels may predict fitness. McGlothlin et al. (2010) found that male dark-eyed juncos with GnRH-induced testosterone levels near (though slightly above) the population mean sired the most within- and extra-pair offspring. These males also had greater survival (McGlothlin et al. 2010), a finding that is in contrast to previous studies on testosterone and survival (e.g., Nolan et al. 1992). Males with higher GnRH-induced testosterone levels produced more within-pair offspring (McGlothlin et al. 2010). Together, these results indicate that stabilizing selection was acting on GnRH-induced testosterone levels through survival

and gains in extra-pair copulations, but positive directional selection was acting on GnRH-induced testosterone levels through within-pair mating success (McGlothlin et al. 2010). It is currently not well-understood how female fitness covaries with GnRH-induced testosterone levels, and if these relationships differ from those of males.

In this study, I used the eastern bluebird (*Sialia sialis*), a common, cavity-nesting songbird, to assess relationships between testosterone levels, reproductive success and survival in males and females. Eastern bluebirds exhibit biparental care in feeding of the young (Grindstaff et al. 2012; Burtka and Grindstaff 2013; Burtka and Grindstaff 2015; Gowaty and Plissner 2015; Burtka et al. 2016) and nest defense (Grindstaff et al. 2012; Burtka and Grindstaff 2013; Burtka and Grindstaff 2015; Burtka et al. 2016). This system allows us to measure hormone levels at multiple time points: within parental as well as aggressive contexts. I used several testosterone and reproductive measurements collected during two consecutive breeding seasons for females and three consecutive breeding seasons for males to assess how individual variation in testosterone levels is related to fitness. Specifically, I tested relationships between initial (pre-GnRH) testosterone levels and GnRH-induced testosterone levels, and clutch size, number of nestlings fledged, and mass of nestlings at fledging. Clutch size and fledging success are commonly used as components of fecundity in birds (Etterson et al. 2011). Mass at fledging is linked to avian reproductive success because it can predict post-fledging survival of offspring (Both et al. 1999; Naef-Daenzer et al. 2001; Wheelright et al. 2003). I also tested for relationships between testosterone measurements and adult body mass because body mass is related to reproductive success in some birds (e.g., Järvinen and Väisänen 1984). Finally, I tested for relationships between testosterone measurements and return rates of

individuals to the breeding population as a proxy for survival (Siefferman and Hill 2007). Because experimental testosterone elevation has been shown to reduce survival while increasing reproductive success, I predicted that return rates would be negatively related to testosterone measurements. Alternatively, if individuals differ primarily in quality, and high quality individuals can increase reproduction without negatively impacting survival, then a positive relationship between return rates and testosterone measurements might be expected (McGlothlin et al. 2010).

METHODS

Morphometric and reproductive measurements

I conducted observations in the field during three breeding seasons (March – August) from 2012 through 2014. I monitored 160 nest boxes across nine bluebird nest box trails in and around Stillwater, Oklahoma, USA, which were checked at least twice per week. I caught adult bluebirds in nest boxes using prop traps. Females were initially trapped for banding near the end of incubation, whereas males were trapped for banding after nestlings reached 4 days of age. At the time of capture, I measured mass of individuals to the nearest 0.1 gram. I marked birds with an aluminum U.S. Fish and Wildlife Service (USFWS) band, and a unique combination of three plastic color bands, which allowed us to determine if an individual bird returned to the population in subsequent years.

For each individual on which I conducted a GnRH challenge (see below), I collected several measurements as proxies for reproductive success: clutch size, number of nestlings fledged, and nestling mass just prior to fledging. In 2012 and 2013, I weighed

nestlings to the nearest 0.1 g when they were 14 days post hatch. In 2014, I weighed nestlings at 15 days post hatch. Bluebird nestling mass increases rapidly early in the nestling phase, but by 13 days, nestling mass begins to asymptote (Pinkowski 1975). Thus, there should not be a large difference in mass between birds weighed on day 14 and birds weighed on day 15.

GnRH challenges within parental and aggressive contexts

In 2012, GnRH challenges were conducted only on males, but in 2013 and 2014, I conducted GnRH challenges on both males and females. I conducted GnRH challenges on 41 total males across three years and 36 total females across two years. I conducted GnRH challenges on 39 males and 35 females within a parental context. Of those birds, 20 males and 13 females were sampled again within an aggressive context. I sampled 3 males and 1 female within the aggressive context, but not within the parental context. I randomly chose which sex within a pair would receive a GnRH challenge, such that only the male or the female was sampled (i.e., I did not perform GnRH challenges on both members of a pair). In 2014, I conducted a brood size manipulation as part of another study. I excluded 10 females and 8 males with manipulated brood sizes from the described analyses on fledging success and nestling mass at fledging in this study. I did not exclude birds with manipulated nests from analyses on the relationship between testosterone levels and clutch size because brood sizes, not clutch sizes, were manipulated.

For GnRH challenges, I first took a blood sample (~50 µl) within three minutes of capturing the bird to measure initial testosterone levels. I then injected the bird with a

standardized dose of GnRH (2.50 mg of chicken GnRH dissolved in 100 µl of phosphate buffered saline) in the pectoralis muscle. I held the bird in a bag for 30 minutes, then took a final blood sample to measure GnRH-induced testosterone levels (Jawor et al. 2006; McGlothlin et al. 2007; DeVries et al. 2012)

I kept blood samples on ice while in the field for up to 3 hours until samples were brought to the laboratory for processing. In the laboratory, I centrifuged samples for 7 minutes at 5000 rpm, and separated the plasma fraction from the red blood cells. Plasma samples were stored at -20º C until analysis.

GnRH challenges were conducted within a parental context and within an aggressive context. For GnRH challenges within a parental context, I observed nestling provisioning behavior by the adults for two hours on two separate days between 0700 and 1100 hours when nestlings were 5–7 days post-hatch. I conducted a GnRH challenge immediately following one randomly chosen parental care observation trial.

For GnRH challenges within an aggressive context, I conducted simulated territorial intrusions between 0700 and 1200 when nestlings were 7–9 days post-hatch. Detailed description of the simulated territorial intrusions is provided in Grindstaff et al. (2012). Briefly, a live, male house sparrow (*Passer domesticus*), a common nest competitor, was placed into a cage which I attached to the focal pair's nest box. The cage was covered during attachment. I retreated to a blind or natural vegetation at least 15 m away and remotely removed the cover from the cage when the bluebird pair was within 100 m of the nest box. I observed aggressive behaviors made by the bluebirds for two minutes (*sensu* Duckworth 2006; Grindstaff et al. 2012). When the trial was over, I

attempted to catch the same bird on which I performed a GnRH challenge within a parental context. I again conducted a GnRH challenge.

Testosterone assay

I quantified testosterone using enzyme immunoassay (EIA) kits (Assay Designs, #901-065), which I had previously optimized for use with eastern bluebirds (*sensu* Wada et al. 2007). Based on the optimization, I diluted plasma samples to 1:30 by adding 10 µl of raw plasma to 290 µl of the assay buffer provided in the kit. I did not add the steroid displacement buffer (SDB) included in the kit. Each diluted sample was run in duplicate on a 96 well microtiter plate. I included five standards of known concentrations (2,000 pg/ml, 500 pg/ml, 125 pg/ml, 31.25 pg/ml, and 7.81 pg/ml) in triplicate to each plate to create the standard curve. I read plates at 405 nm on a Biotek ELx808 microplate reader. The intra-assay coefficient of variation was 8.9% and the inter-assay coefficient of variation was 11.7% (N=11 plates).

Statistical analyses

All statistical modeling was performed in R version 3.2.3 (http://www.r-project.org). Testosterone values were not normally distributed, so they were natural log transformed. Males and females were analyzed separately, and all model residuals were checked for normality. Many individuals had multiple hormone measurements across behavioral contexts or years so I used linear mixed effects models that all included an individual identifier (band number) as a random effect.

I first determined how initial and GnRH-induced testosterone levels predicted clutch size and the number of offspring fledged, when controlling for breeding date, and year. I created generalized linear mixed models (GLMMs) with a Poisson error structure (lme4 package; Bates et al. 2015) with initial testosterone levels, GnRH-induced testosterone levels, breeding date, and year as fixed effects, individual identity as a random effect, and either clutch size or number of offspring fledged as response variables. I next determined how initial and GnRH-induced testosterone levels predicted nestling mass at fledging and adult mass, when controlling for breeding date and year. I created GLMMs with a Gaussian error structure (lme4 package; Bates et al. 2015) with initial testosterone levels, GnRH-induced testosterone levels, breeding date, and year as fixed effects, individual identity as a random effect, and either nestling mass at fledging or adult mass as response variables.

My data on return rates were coded as 0 if a bird had bred in the population during only one year, and 1 if a bird had bred in the population during more than one year (I included birds that were banded up to three years prior to the beginning of the study and up to one year after the study concluded). For each testosterone measurement, I used GLMM with a binomial error structure (lme4 package; Bates et al. 2015) and individual identity as a random effect to determine if initial testosterone levels or GnRH-induced testosterone levels predicted whether a bird would return to the population. I ran separate analyses for males and females.

RESULTS

Do adult testosterone levels predict clutch size or fledging success?

Male untransformed initial testosterone levels ranged from 0.04–4.25 ng/ml (mean ± SE = 0.93 ± 0.09 ng/ml) and untransformed GnRH-induced testosterone levels ranged from 0.05–5.22 ng/ml (mean ± SE = 1.48 ± 0.15 ng/ml). Female untransformed initial testosterone levels ranged from 0.07–4.33 ng/ml (mean ± SE = 0.98 ± 0.11 ng/ml) and untransformed GnRH-induced testosterone levels ranged from 0.15–3.17 ng/ml (mean ± SE = 0.80 ± 0.09 ng/ml). Male GnRH-induced testosterone levels were significantly higher than initial levels (mixed model: $F_{1,37}$=18.4, P<0.0001). Female GnRH-induced testosterone levels were slightly, but marginally significantly lower than initial testosterone levels (mixed model: $F_{1,31}$=4.00, P=0.05).

For males, their partner's clutch sizes were not related to their initial testosterone levels or their GnRH-induced testosterone levels (Table 1). For females, clutch size was not related to initial testosterone levels or GnRH-induced testosterone levels (Table 2). For males, the number of offspring fledged was not related to initial testosterone levels or GnRH-induced testosterone levels (Table 1; Fig. 1a). For females, the number of offspring fledged was not related to initial testosterone levels or GnRH-induced testosterone levels (Table 2; Fig. 1b).

Do adult testosterone levels predict nestling mass at fledging?

For males, nestling mass at fledging was not related to initial testosterone levels, but had a non-significant tendency to increase with increasing GnRH-induced

testosterone levels (Table 1; Fig. 2a). For females, nestling fledging mass was not significantly related to GnRH-induced testosterone levels (Table 2; Fig. 2b).

Do adult testosterone levels predict adult mass?

For males, individuals that were heavier had higher initial testosterone levels and higher GnRH-induced testosterone levels (Table 1; Fig. 3). I also tested if clutch size, fledging success, and nestling mass were related to male body mass. There was no relationship between number of eggs laid by the male's partner, fledging success, or nestling mass at fledging and male mass (clutch size: $F_{1,37}$=1.40, P=0.24; fledging success: $F_{1,37}$=1.59, P=0.23; fledging mass: $F_{1,31}$=0.40, P=0.53).

For females, body mass was not related to initial testosterone levels or GnRH-induced testosterone levels (Table 2). There was a marginally significant negative relationship between female mass and clutch size such that females that laid more eggs were lighter ($F_{1,31}$=4.00, P=0.05). Females that fledged more young were significantly lighter ($F_{1,24}$=4.70, P=0.04). There was no significant relationship between nestling mass at fledging and female mass (fledging mass: $F_{1,24}$=0.58, P=0.45).

Do testosterone levels predict adult return rates?

Male initial testosterone levels and GnRH-induced testosterone levels did not predict return rates (initial, GLMM: P=0.69; GnRH-induced, GLMM: P=0.75). Female initial testosterone levels and GnRH-induced testosterone levels also did not predict return rates (initial, GLMM: P=0.72; GnRH-induced, GLMM: P=0.50)

DISCUSSION

In this study, I sought to determine if individual variation in testosterone levels was predictive of reproductive success in male and female eastern bluebirds. To capture individual variation in testosterone production capabilities, I measured initial testosterone levels and testosterone levels produced in response to administration of GnRH (GnRH-induced testosterone levels). For males, clutch sizes of their mates were not related to their testosterone concentrations. I measured testosterone during the nestling period, but if I had instead quantified male testosterone when their mates were fertile, I might have seen different results. In many birds, elevated testosterone often increases courtship (De Ridder et al. 2000; Wiley and Goldizen 2003). In dark-eyed juncos, testosterone can influence attractiveness to females (Enstrom et al. 1997), which may increase mating success. Females can adjust investment in eggs in relation to mate attractiveness by altering clutch size (Horváthová et al. 2012) and size of individual eggs (Cunningham and Russell 2000; Horváthová et al. 2012). We might then expect males with higher testosterone levels to be mated to females that produced more or larger eggs. Future studies on both male and female testosterone levels and how they relate to traits such as fecundity and attractiveness, will likely benefit by taking multiple samples across the entire breeding period from both sexes.

Clutch size was not related to female initial testosterone levels. Compared to studies on males, relatively few studies have investigated female testosterone levels in relation to reproductive success. Some previous studies suggest that high levels of testosterone in females can negatively impact egg-laying (e.g., Rutkowska et al. 2005; Gerlach and Ketterson 2013), but these studies were on females with artificially elevated

testosterone levels. In these studies, female testosterone levels were manipulated prior to or during egg-laying (Rutkowska et al. 2005; Gerlach and Ketterson 2013), whereas in my study, females were sampled during the nestling rearing stage of the nesting cycle. I did not find the predicted negative relationship between testosterone levels and clutch size in my study. One limitation of my study is that I conducted GnRH challenges only during the nestling period, not during egg-laying. In female dark-eyed juncos, individuals were most responsive to GnRH just prior to clutch initiation, but did not respond with significantly elevated testosterone levels at other times during the nesting cycle (Jawor et al. 2007). If I had sampled female bluebirds during egg-laying, I might have found a different relationship between clutch size and levels of initial or GnRH-induced testosterone.

For both sexes, fledging success was not related to testosterone levels. In both sexes, it might be expected that high baseline testosterone levels to be associated with reduced parental behaviors (Wingfield et al. 1990; O'Neal et al. 2008), so birds with higher testosterone levels may fledge fewer young (Hegner and Wingfield 1987). However, previous work in our population has found that male and female nest visit rates are not associated with baseline total androgen levels, GnRH-induced testosterone levels, or testosterone production (Burtka et al. 2016; Ambardar and Grindstaff, unpubl. data). My results also confirm previous findings in our population that demonstrated that fledging success was not related to baseline androgen concentrations in males or females (Burtka et al. 2016). Extra-pair young were not quantified in this study, but extra-pair matings can increase male reproductive success, and in other bluebird populations 24–26% of nests have at least one extra-pair offspring (Meek et al. 1994; Stewart et al.

2010). In a study on dark-eyed juncos, McGlothlin et al. (2010) showed evidence of natural selection acting on GnRH-induced testosterone levels using within- and extra-pair reproductive success, and survival as fitness measurements. Partitioning my data into within- and extra-pair young might reveal a results similar to those in McGlothlin et al. (2010). Return rates, a proxy for survival, also were not related to bluebird testosterone concentrations. Therefore, I cannot conclude that selection was acting on testosterone levels via reproductive success or increased survival as in McGlothlin et al. (2010).

For males, body mass was positively related to initial testosterone levels and GnRH-induced testosterone levels. Experimental elevation of testosterone through the use of implants has been associated with reduced body mass in some studies (Ketterson et al. 1991; Ros et al. 1997), suggesting a potential cost of high testosterone levels. However, Hunt et al. (1999) found that testosterone implants increased mass in Lapland longspurs (*Calcarius lapponicus*). If gonad size is positively correlated with body mass, then larger males might have been better able to produce and maintain higher testosterone levels. In male yellow-pine chipmunks (*Tamias amoenus*), body mass was positively correlated with gonad size (Schulte-Hostedder and Millar 2004), and gonad mass has been shown to be an important predictor of individual variation in testosterone levels (Bergeon Burns et al. 2014).

For females, neither initial nor GnRH-induced testosterone levels predicted body mass. Experimental elevation of testosterone has been shown to decrease body mass in female dark-eyed juncos (Clotfelter et al. 2004). This response was similar to that of male juncos (Nolan et al. 1992). Selection for higher levels of testosterone in males might initially be accompanied by a correlated, but costly response in females. In bluebirds,

female relationships between mass and testosterone levels were not similar to those of males, so I explored the potential contribution of clutch size to female body mass. Females that produced larger clutches and fledged more young were lighter, which might mean that more body mass was lost while producing larger clutches and raising more young.

In addition to phenotypic engineering, it is important to consider natural variation in hormonal measurements, particularly beyond baseline levels. It is also important to consider relationships between testosterone levels and fitness in both sexes. I this study, I did not find evidence of a positive association between reproductive success and GnRH-induced testosterone levels in male and female bluebirds. Based on these patterns, I cannot conclude that selection might be acting on either initial or GnRH-induced testosterone levels in this population. The relationships between fitness and testosterone in both sexes are complex and may vary among species. A combination of experimental and correlative studies will likely be the best approach to develop large-scale patterns of testosterone-fitness relationships for males and females.

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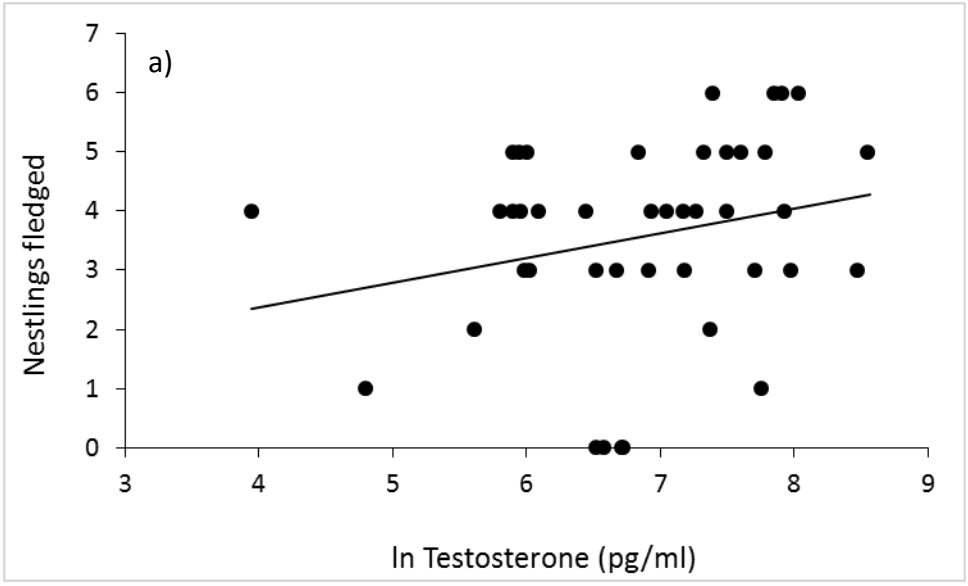
Table 1. Results of models to predict partner's clutch size, fledging success, nestling mass at fledging, and adult body mass for male bluebirds. Models are mixed models fitted with ML estimations with individual identity as a random effect. Models to predict clutch size and number of offspring fledged were fitted with Poisson errors, and models to predict nestling mass and adult mass were fitted with Gaussian errors.

Response	Parameter	F	df	P
Clutch size	Initial testosterone	0.09	1,37	0.91
	GnRH-induced testosterone	0.36	1,37	0.86
	Breeding date	1.08	1,37	0.32
	Year	<0.01	1,37	0.94
N offspring fledged	Initial testosterone	0.50	1,31	0.44
	GnRH-induced testosterone	0.32	1,31	0.89
	Breeding date	1.49	1,31	0.38
	Year	2.81	1,31	0.09
Nestling mass	Initial testosterone	1.63	1,28	0.21
	GnRH-induced testosterone	3.18	1,28	0.06
	Season	0.48	1,28	0.47
	Year	9.35	1,28	**<0.01**
Adult mass	Initial testosterone	9.87	1,37	**<0.01**
	GnRH-induced testosterone	6.01	1,37	**0.03**
	Season	<0.01	1,37	0.97
	Year	48.18	1,37	**<0.01**

Table 2. Results of models to predict clutch size, fledging success, nestling mass at fledging, and adult body mass for female bluebirds. Models are mixed models fitted with ML estimations with individual identity as a random effect. Models to predict clutch size and number of offspring fledged were fitted with Poisson errors, and models to predict nestling mass and adult mass were fitted with Gaussian errors.

Response	Parameter	F	df	P
Clutch size	Initial testosterone	0.06	1,31	0.96
	GnRH-induced testosterone	0.47	1,31	0.73
	Breeding date	0.62	1,31	0.43
	Year	0.03	1,31	0.87
N offspring fledged	Initial testosterone	0.45	1,24	0.74
	GnRH-induced testosterone	0.79	1,24	0.80
	Breeding date	2.80	1,24	0.10
	Year	<0.01	1,24	0.95
Nestling mass	Initial testosterone	0.07	1,21	0.78
	GnRH-induced testosterone	0.02	1,21	0.94
	Breeding date	0.43	1,21	0.45
	Year	2.85	1,21	0.09
Adult mass	Initial testosterone	0.48	1,31	0.47
	GnRH-induced testosterone	1.52	1,31	0.26
	Breeding date	2.76	1,31	0.11
	Year	6.42	1,31	**0.02**

Figure 1. Relationship between GnRH-induced testosterone levels and fledging success in a) males and b) females. Male and female GnRH-induced testosterone levels were positively related to the number of offspring fledged (males: r^2=0.14, P=0.03; females: r^2=0.17, P=0.049). Results are from mixed models with adult identity as a random effect. Points on figure are GnRH-induced testosterone levels averaged across behavioral contexts for each individual.



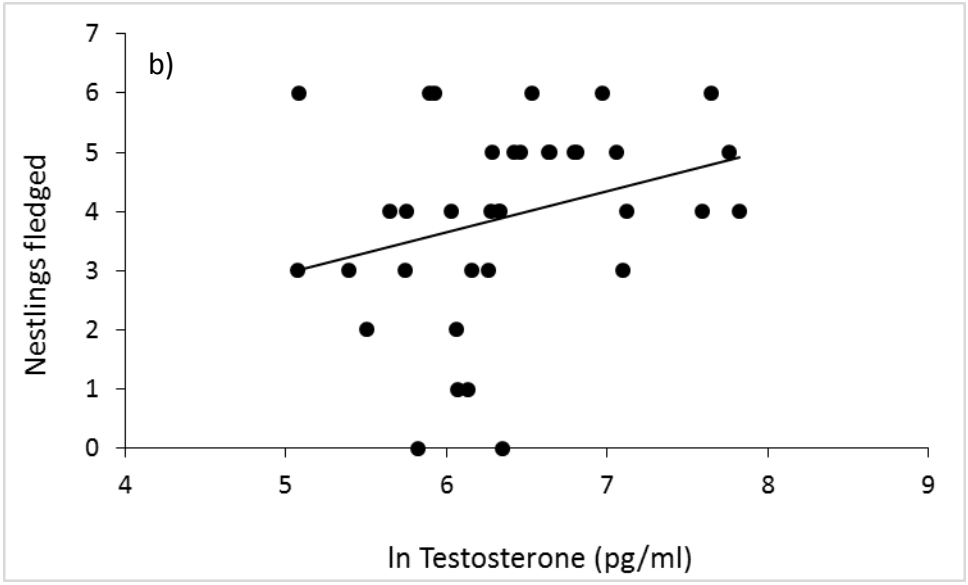


Figure 2. Relationship between GnRH-induced testosterone levels and nestling mass at fledging in a) males and b) females. Male GnRH-induced testosterone levels tended to be positively related to nestling mass (r^2=0.32, P=0.09). Female GnRH-induced testosterone levels were not related to nestling mass (r^2=0.16, P=0.76). Results are from mixed models with adult identity as a random effect. Points on figure are GnRH-induced testosterone levels averaged across behavioral contexts for each individual, and the average nestling mass within each nest.

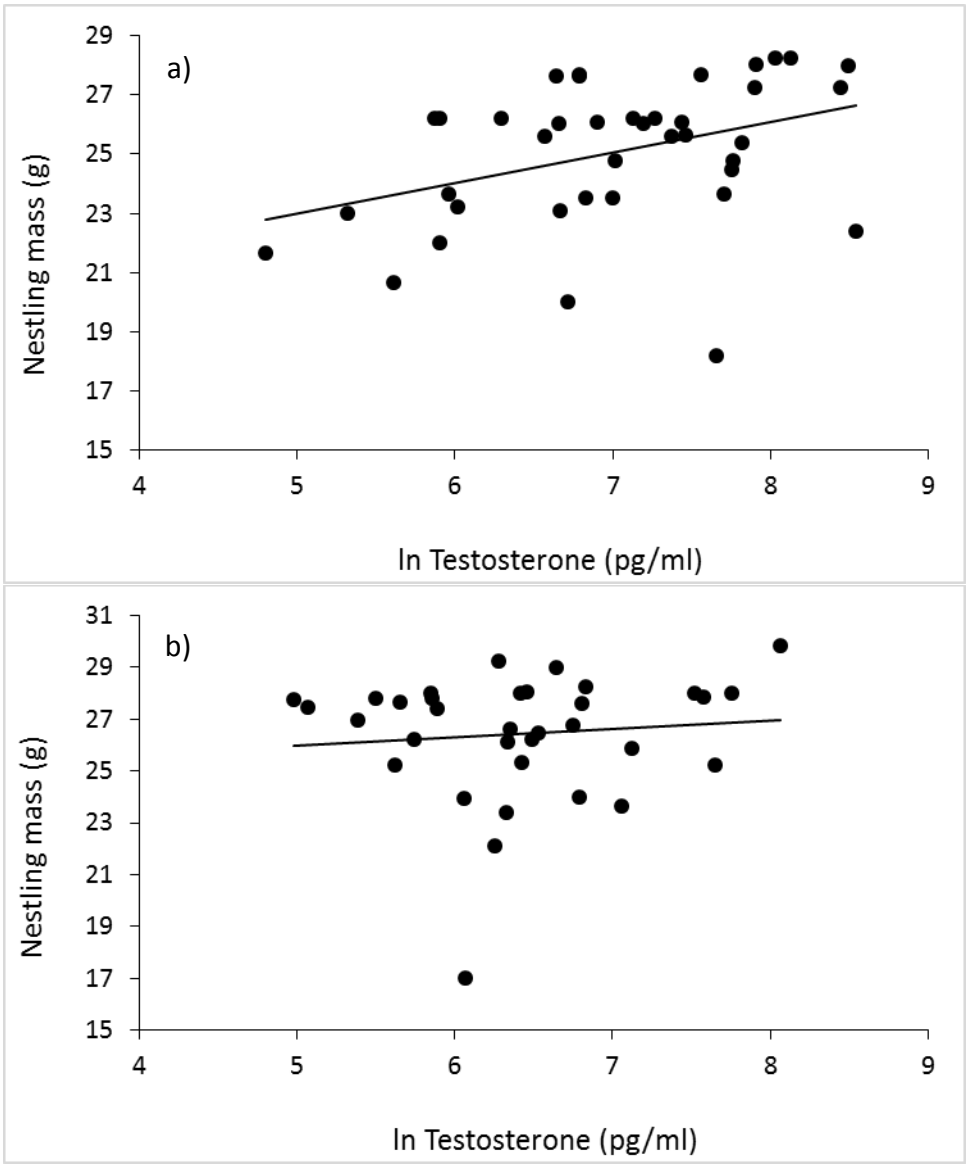
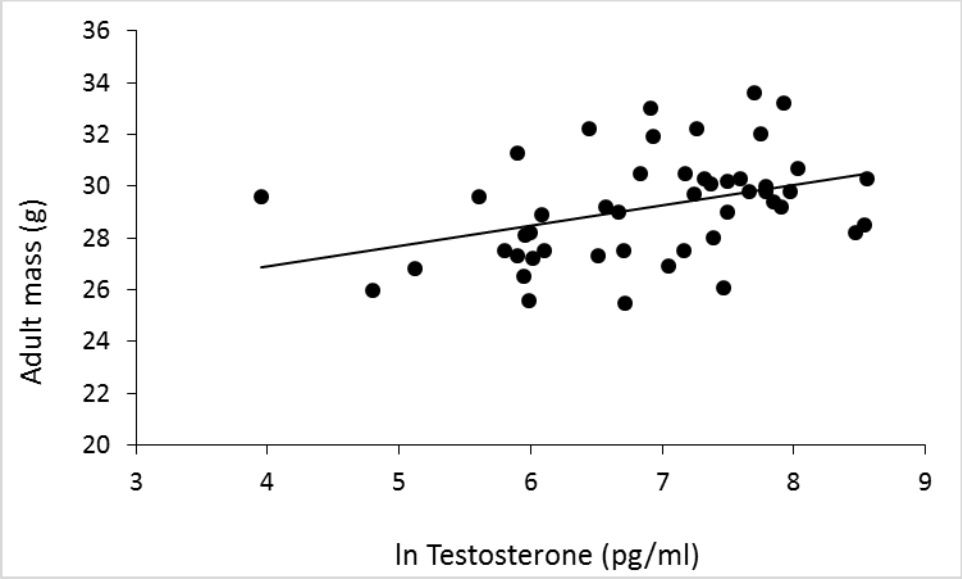


Figure 3. Relationship between GnRH-induced testosterone levels and adult body mass in males. Male GnRH-induced testosterone levels were positively related to body mass (r^2=0.10, P=0.02). Results are from mixed models with adult identity as a random effect. Points on figure are GnRH-induced testosterone levels averaged across behavioral contexts for each individual.



CHAPTER IV

REARING ENVIRONMENT AFFECTS PLUMAGE COLORATION BUT NOT TELOMERE LENGTHS IN NESTLING EASTERN BLUEBIRDS (*SIALIA SIALIS*)

INTRODUCTION

Early life experience can have important and long-lasting consequences (Monaghan et al. 2012). One potential source of early life stress for young animals is increased brood size (Naguib and Gil 2005) because it can mean reduced food delivery by parents to offspring (Saino et al. 1997; Leonard et al. 2000; Siefferman and Hill 2007a) and increased competition among siblings (Kacelnik et al. 1995). In birds, clutch (and thus, brood) size is limited by the ability of parents to provide care to offspring (Lack 1947), but parents can also incur costs when they increase effort for larger broods (Monaghan and Nager 1997). If brood size is within the optimal range that has evolved for a given species, parents might be able to ameliorate the effects of large brood size on offspring by providing more care to young. If parents do not provide increased care for larger broods, then offspring may incur heavy costs.

The costs to offspring of growing up in a large brood are effectively illustrated in brood size manipulation studies, where brood size is experimentally increased and/or decreased. When adults reduce per nestling feeding rates to large broods, young fledge at

lower body weight (Saino et al. 1997; Siefferman and Hill 2007a) because parental feeding rates are often associated with offspring growth rates and fledging mass (Ardia 2007). Fledging at a reduced body mass is particularly detrimental to young because mass at fledging in birds is a good predictor of post-fledging survival and recruitment (Both et al. 1999; Naef-Daenzer et al. 2001; Wheelright et al. 2003).

Another potential cost to offspring of growing up in a large brood is increased exposure to the stress hormone, corticosterone. During the acute stress response, corticosterone is produced at elevated levels when the hypothalamic-pituitary-adrenal axis is activated in response to a stressor (Romero and Butler 2007). Being raised in an enlarged brood has been shown to elevate baseline corticosterone levels in nestling barn swallows (*Hirundo rustica*; Saino et al. 2003), which can potentially have lasting impacts. For example, exposure to corticosterone early in life can suppress growth and alter adult behavior in birds (Spencer and Verhulst 2007), and in humans, stress early in life can impair cognitive and emotional functions (Pechtel and Pizzagalli 2011). Increased competition or crowding in large broods might elevate corticosterone levels in nestlings raised in those broods (Saino et al. 2003; Eraud et al. 2008). Reduced provisioning by parents might be another factor that can elevate corticosterone levels because food stress is known to elevate baseline and stress-induced corticosterone levels (Kitaysky et al. 1999; Saino et al. 2003; Kitaysky et al. 2006; Rensel et al. 2010). Alternatively, if parents adjust their feeding rates for enlarged broods (Neuenschwander et al. 2003), enlarged brood size may not be reflective of a "poor" environment and may not have an impact on nestling corticosterone levels (Lobato et al. 2008).

A recently appreciated cost to offspring of growing up in a large brood is a reduction in telomere length (Boonekamp et al. 2014). Telomeres are non-coding, repeating sequences of DNA that are found at the ends of chromosomes (Blackburn 1991) and appear to aid in prevention of chromosome degradation (Angelier et al. 2013). Importantly, exposure to experimentally elevated corticosterone early in life resulted in accelerated telomere loss in young European shags (*Phalacrocorax aristotelis*; Herborn et al. 2014). It is possible that high levels of corticosterone associated with enlarged brood sizes may be a cause of increased telomere loss. Accelerated telomere shortening may impact survival because once telomeres have been reduced to a critical length, they are no longer functional (Monaghan and Haussmann 2006). This typically results in either cell death (apoptosis) or replicative senescence (Monaghan and Haussmann 2006). In fact, telomere length and telomere loss early in life can predict lifespan (Heidinger et al. 2012). In jackdaws (*Corvus monedula*), nestlings raised in enlarged broods had greater telomere loss than nestlings in reduced broods (Boonekamp et al. 2014). This loss experienced early in life had long lasting effects as it was predictive of telomere lengths in adulthood (Boonekamp et al. 2014).

Being raised in an enlarged brood can also impact the development of important sexual ornaments, such as feather coloration. Brood size manipulations using great tits (*Parus major*; Jacot and Kempenaers 2007) and eastern bluebirds (*Sialia sialis*; Siefferman and Hill 2007a) have shown that nestlings raised in enlarged broods have less ornamented plumage. Sexual ornaments are a costly investment that are hypothesized to signal individual quality (Zahavi 1975; Hamilton and Zuk 1982). Feather color in birds is one of the most elaborate displays of ornamentation (Hill and McGraw 2006). In adult

birds, feather color has been linked to body condition (Grindstaff et al. 2012), reproductive success (Siefferman and Hill 2003; Safran and McGraw 2004), and social status (Vitousek et al. 2013). In young birds, feather color can carry important signaling functions and may influence investment by parents (Ligon and Hill 2010). Experimental studies in great tits and in eastern bluebirds have demonstrated that manipulating feather color alters parental food allocation to young (Galvan et al. 2008; Tanner and Richner 2008; Ligon and Hill 2010). Feather color in nestlings may also be an indicator of condition, such that ornamentation reflects the quality of the rearing environment (Siefferman and Hill 2007a).

In this study, I conducted a brood size manipulation to assess the effects of rearing environment on nestling eastern bluebirds. In particular, I was interested in investment by the parents and potential costs incurred by nestlings in enlarged broods. I quantified adult provisioning behavior of offspring and defense of the nest as measures of parental investment. I quantified offspring body mass, growth rates, corticosterone levels, telomere length, and plumage coloration to track the impacts of brood size. I also assessed the relationships among these variables. To my knowledge, associations between feather color and telomere length have not been previously explored in young birds. I hypothesized that decreased investment (i.e., lower feeding rates and reduced nest defense intensity) by adults of enlarged broods would lead to the following costs for nestlings: 1) reduced mass and slower growth rates, 2) higher baseline and stress-induced corticosterone levels, 3) shorter telomeres and greater telomere loss, and 4) less ornamented feathers. Alternatively, parents might be able to compensate for large brood sizes by adjusting their feeding rates. In this case, adults may feed enlarged broods more

often overall, making the per nestling feeding rates similar across brood size groups. Under this scenario, rearing environments would be similar in quality, and I would not predict differences in nestling mass, corticosterone levels, telomere lengths or feather coloration among brood size groups.

METHODS

Study species

Eastern bluebirds commonly nest in human-made nest boxes (Gowaty and Plissner 2015) and brood sizes in our population range from 3–6 nestlings. Hatching is synchronous within broods, typically occurring on the same day for a given brood (personal obs.). Adults provide biparental care to the young in the form of food provisioning, and nest defense in response to competitors, behaviors that are easy to quantify in our population (Grindstaff et al. 2012; Burtka and Grindstaff 2013; Burtka and Grindstaff 2015; Burtka et al. 2016). Bluebird nestlings develop sexually dimorphic plumage coloration early in life. By the time nestlings fledge (16–20 days after hatching), male nestlings are more ornamented on the wing and tail feathers. The bright blue feathers are structural colors which reflect in the ultraviolet (UV) spectrum (Grindstaff et al. 2012). Nestling rump feathers, which are not as colorful to the human eye, also reflect in the UV spectrum (Grindstaff et al. 2012). As adults, the feathers on the breast are a rusty red coloration (Pyle 1997). Nestlings also begin to develop this melanin-based breast coloration before they fledge (Gowaty and Plissner 2015), though their feathers are spotted with white and less red than adult feathers.

Fieldwork

I conducted observations and experiments in the field during the 2014 breeding season (March–August). I monitored 160 nest boxes across nine bluebird nest box "trails" in and around Stillwater, Oklahoma, USA. Each nest box trail consists of a series of nest boxes that were checked at least twice per week. I checked nests daily around the projected hatch date, and the actual hatch date was recorded as nestling day 0.

Brood size manipulation and nestling measurements

When nestlings were two days old, I moved 1–2 nestlings between nests with the same hatch date to create enlarged broods (mean ± SE nestlings = 5.70 ± 0.15, N = 10) and reduced broods (mean ± SE nestlings = 3.25 ± 0.16, N = 9). I left some nests unmanipulated, but did remove two nestlings from the nest for a similar amount of time as nestlings from manipulated nests, then returned them to their original nest. I used these nests as controls (mean ± SE nestlings = 3.83 ± 0.39, N = 12). On nestling day 2, prior to moving nestlings, I painted each nestling's nails with nail polish so I could distinguish between individuals. When nestlings were 11 days old, I banded each one with a USFWS aluminum band with a unique number combination.

I weighed nestlings to the nearest 0.1 g on day 2 prior to manipulation. Day 2 mass did not differ across brood size groups for males ($F_{2,32}$=0.09, P=0.91) or females ($F_{2,32}$=0.30, P=0.74). I weighed nestlings again at days 5, 11, and 15, just before fledging. Mass of nestling bluebirds increases rapidly between hatching and 11 days post hatching, but reaches an asymptote at around day 13 (Pinkowski 1975). Mass at day 15 is therefore a close estimate of fledging mass. I also sexed nestlings on day 15. Eastern bluebird

nestlings can be sexed based on plumage coloration when they are near fledging, with males having more colorful wing and tail feathers than females (Gowaty and Plissner 2015).

Parental care

I videotaped provisioning of nestlings by the adult bluebirds between nestling days 5–7. I recorded on two separate days between 0700 and 1200 with a Sony HDR CX260 digital video camera on a tripod at least 10 m from the nest box. I recorded for at least two hours and fifteen minutes. When I analyzed the videos, I recorded the latency to the first nest box visit, then recorded subsequent nest visits and time spent in the nest box for two hours after the initial nest visit.

Nest defense behavior

I observed parental defense of the offspring using simulated territorial intrusions between 0700 and 1200 on all pairs between nestling days 7–9. Detailed methods describing simulated territorial intrusions are described in Grindstaff et al. (2012). Briefly, a live, male house sparrow (*Passer domesticus*), a common nest competitor, was placed into a cage that I attached to the focal pair's nest box. The cage was covered during attachment. I retreated to a blind or natural vegetation at least 15 m away and remotely removed the cover from the cage when the bluebird pair was within 100 m of the nest box. I observed the birds for two minutes and recorded the number of times male and female bluebirds hovered within 0.5 m of the cage, landed on the cage, and attempted to attack the sparrow within the cage (*sensu* Duckworth 2006; Grindstaff et al. 2012). I

used these responses to calculate an aggression score for each bird. Aggression scores ranged from 1–6, with 6 being the most aggressive (Duckworth 2006).

Nestling blood sampling

I collected two blood samples from each nestling. I collected the first blood sample (~5 µl) on nestling day 2. Whole blood was stored in approximately 10 µl of phosphate buffered saline (PBS) and kept on ice while in the field. During blood sampling, nestlings were kept warm from the investigator's body heat or chemical hand warmers. I returned nestlings to either their original or new nest immediately after blood sampling (mean time out of nest ± SE = 27.6 ± 1.89 minutes, range: 11–82 minutes).

I collected the second blood sample on nestling day 15. I removed one nestling at a time from the nest and collected a blood sample (100 µl). I stored each sample in a tube coated with heparin, an anticoagulant that facilitates the process of separating the plasma from the red blood cells. Tubes were kept on ice while in the field. I sampled the first nestling from each nest within 3 minutes of initial disturbance.

Whole blood samples in PBS from 2 day old nestlings were stored at -80 ºC. Day 15 blood samples were centrifuged at 5000 rpm for 7 minutes to separate the plasma from the red blood cells. I removed the plasma fraction, which was used for measuring corticosterone levels, and stored it in a fresh tube, which was kept at -20 ºC. The red blood cells, which were used for measuring telomere lengths, remained in the original tube and were stored at -80 ºC. The volume of day 2 whole blood samples was insufficient to separate out the plasma fraction, and instead whole blood stored in PBS was used to measure telomere lengths.

Corticosterone assay

I quantified corticosterone using enzyme immunoassay (EIA) kits (Assay Designs, #901-097) optimized for use with eastern bluebird plasma (*sensu* Wada et al. 2007). Based on the optimization, I added 10 µl of 1.5% steroid displacement buffer (SDB) from the kit to 10 µl of raw plasma. Five minutes later, I added 380 µl of assay buffer provided in the kit to dilute samples to 1:40. Each diluted sample was run in duplicate on a 96 well microtiter plate. I included five standards of known concentrations (20,000 pg/ml, 4,000 pg/ml, 800 pg/ml, 160 pg/ml, and 32 pg/ml) in triplicate. I read plates at 405 nm on a Biotek ELx808 microplate reader. The intra-assay coefficient of variation was 6.6% and the inter-assay coefficient of variation was 9.2% (N=4 plates).

Telomere assay

Whole blood samples collected on day 2 and red blood cells reserved from blood sampling on day 15 were used to measure bluebird nestling telomere lengths. For day 15 samples, I extracted DNA using 6–8 µl of red blood cells in 194–192 µl of PBS (for a total volume of 200 µl) using Machery-Nagel NucleoSpin Blood kits (Machery-Nagel, #740951.250). For day 2 samples, I added 190 µl of PBS directly to the collection tube with the whole blood in PBS to bring the total volume to 200 µl. Following the kit instructions, I lysed the sample by adding 25 µl of proteinase K and 200 µl of lysis buffer, then vortexed the samples for 10–20 s. I incubated samples at 70 ºC for 30–45 minutes. I added 200 µl of 100% ethanol to each sample to adjust DNA binding conditions. I then pipetted the entire sample into a spin column provided with the kit. The

spin column had a silica membrane that collected the sample, allowing other reagents to pass through when centrifuged. I centrifuged the sample for 1 minute at 11,000 x g. I washed the silica membrane of the spin columns twice with 500 µl of wash buffer for the first wash and 600 µl of wash buffer for the second wash, centrifuging for 1 minute at 11,000 x g between washes. I dried the silica membrane by centrifuging the spin column again, and then eluted the DNA by adding elution buffer that was preheated to 70 ºC to the silica membrane. I incubated the sample at room temperature for 3 minutes, and then centrifuged the spin column for 1 minute at 11,000 x g, which caused the elution buffer to draw the DNA through the silica membrane into a collection tube for storage. For day 15 samples, I used 100 µl of elution buffer and for day 2 samples I used 30 µl of elution buffer. I measured DNA yield and purity with a NanoDrop 1000 spectrophotometer. If extracted samples had 260/280 ratios lower than 1.7 or 260/230 ratios lower than 1.8, I re-extracted DNA from those samples.

I used a Stratagene Mx3005P quantitative PCR (qPCR) machine to measure telomere length, represented as the T/S ratio, which is the ratio of telomere repeat copy number (T) to single copy gene number (S) from a particular sample relative to a reference sample (Heidinger et al. 2012). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the single copy gene (Heidinger et al. 2012).

I ran separate plates for telomere and GAPDH reactions, and each reaction was run in triplicate with 6 µl of DNA. I used the following primers to amplify telomere and GAPDH sequences: Telomere forward tel1b (5’-CGG TTT GTT TGG GTT TGGGTT TGG GTT-3’), and reverse tel2b (2b 5’-GGC TTG CCT TAC CCT TACCCT TAC CCT-3’); Zebra finch GAPDH forward (5’-CAT CAC AGC CAC ACA GAA GA-3’), and

reverse (5'-CTC CAG TAG ATG CTG GGA TAA TG-3') (Heidinger et al. 2012). Primer concentrations for forward tel1b and reverse tel2b were both 200 nM. Primer concentrations were also 200 nM for forward GAPDH and reverse GAPDH. I mixed primers with 12.5 µl of SYBR green qPCR SuperMix for a total volume of 19 µl (Heidinger et al. 2012). I used the following cycling parameters for telomere qPCR reactions: 15 minutes at 95 ºC, followed by 27 cycles of 15 seconds at 95 ºC, 30 seconds at 58 ºC, and 30 seconds at 72 ºC (Heidinger et al. 2012). I used the following cycling parameters for GAPDH qPCR reactions: 15 minutes at 95 ºC, followed by 40 cycles of 15 seconds at 95 ºC, 30 seconds at 60 ºC, and 30 seconds at 72 ºC (Heidinger et al. 2012).

I included on each plate a reference sample that was serially diluted from a bluebird nestling extraction to create a standard curve (40, 20, 10, 5, 2.5 ng) and to measure reaction efficiencies (Heidinger et al. 2012). Mean efficiencies for telomeres and GAPDH were 92.5% and 93.6% respectively.

Nestling feather collection and color analysis

When nestlings were 15 days old, I pulled nine chest feathers and nine rump feathers from the same spot on each individual. I stored feathers in archival envelopes that were protected from light. I taped feathers to black paper (Canson color #425, Stygian black) by overlapping the feathers on each other to mimic how they would appear on the bird's body (Siefferman and Hill 2003; Grindstaff et al. 2012). I used a UV-Vis spectrometer (USB 4000, Ocean Optics) with a deuterium-halogen lamp (DH-2000-BAL, Ocean Optics) and a probe (QR400-7-SR-BX, Ocean Optics) fitted with a sheath to exclude ambient light (Grindstaff et al. 2012). To measure color reflectance

spectra, the probe was held 5 mm from each feather patch at a 90º angle to the feathers and read into the computer using SpectraSuite software (Ocean Optics). I recorded reflectance values relative to a white standard (WS-1-SL diffuse reflectance standard, Labsphere) and a black standard (SpyderCube). I took five measurements of each feather patch, lifting the probe between each measurement. The one investigator taped all of the feathers, while two investigators collected color measurements together. The same investigator held the probe for all measurements. Repeatability was high ($r > 0.74$); Nakagawa and Schielzeth 2010) for both rump and chest measurements. As nestlings, the coloration on the rump feathers is fairly uniform, but the chest feathers have white and reddish spots. When taking measurements of chest feathers, the investigator holding the probe attempted to hold it over an area where the reddish color was present.

I used the program CLR5 version 1.05 (Montgomerie 2008) to calculate brightness, saturation, and hue for each feather patch (Grindstaff et al. 2012). Brightness is a measurement of how much light is reflected by the feather, and is calculated by summing the total reflectance from 320 to 700 nm. I used the brightness metric given by CLR5 (B1) for chest and rump feathers. Saturation is a measurement of how much light is reflected at specific color wavelengths in relation to the entire spectrum. Saturation for red chest feathers (S1R in CLR5) was the ratio of reflectance in the red wavelength range (575–700 nm) to the reflectance of the full spectrum (320–700 nm) (Montgomerie 2006; Grindstaff et al. 2012). Rump feather saturation was the ratio of reflectance of blue (S1B; 450–475 nm), violet (S1V; 400–450 nm), and ultra-violet (S1U; 320–300 nm) wavelength ranges to full spectrum reflectance (320–700 nm) (Montgomerie 2006; Grindstaff et al. 2012). Hue is a measurement of the actual color of the feather. For rump

feather hue, I used the CLR5 metric, H1. The wavelength was calculated as the peak reflectance wavelength. I did not calculate hue for chest feathers, because it does not vary much among individuals (Siefferman et al. 2005; Grindstaff et al. 2012).

Statistical analyses

All statistical modeling was performed in R version 3.2.3 (http://www.r-project.org). Corticosterone values were not normally distributed, so they were natural log transformed. All model residuals were checked for normality. I first used linear models to test the effect of brood size group on the number of nest box visits adult male and female bluebirds made during the two hour observation period, and on adult male and female aggression scores. I also analyzed the number of nest visits per nestling per two hours to determine the effect of brood size on parental care provided to each individual nestling.

To determine how brood size group affected nestling mass at fledging, I used the nlme package (Pinheiro et al. 2016) to create a mixed model with brood size group and date as fixed effects, manipulated nest ID and natal nest ID as random effects, and mass at day 15 as the response variable. I ran separate analyses for male and female nestlings

I also examined the effect of brood size group on nestling growth rates. Nestling growth from hatching to fledging typically follows a sigmoidal pattern (Ricklefs 1968; Ricklefs 1973). To calculate growth rates, I first modeled nestling growth using the self-starting Gompertz function, SSgompertz, in R. SSgompertz uses the following parameterization:

$$mass = a_0 exp(-b_0 b_1^{age}),$$

which I interpreted following methods in Sockman et al. (2008). I analyzed the parameters by plotting different Gompertz curves, varying one parameter (e.g., a_0), while holding the other two parameters (e.g., b_0 and b_1) fixed at means that were derived from fitting the Gompertz function to individual nestlings (Sockman et al. 2008). Similar to Sockman et al. (2008), I determined that a_0 sets the height of the asymptote (Fig. 1), which can be interpreted as day 15 mass, b_0 sets the y-intercept (Fig. 2), and is interpreted as day 2 mass, and b_1 sets the inflection point as well as the growth rate (Fig. 3). I used b_1 values obtained for individual nestlings as a measurement of growth rate. This parameter was used as the response variable in a mixed model that had treatment and date as fixed effects, and manipulated nest ID and natal nest ID as random effects. I ran separate models for male and female nestlings.

For corticosterone measurements, I ran separate linear models for the first and third nestlings sampled at each nest. When collecting blood samples on day 15 to measure corticosterone, I was unable to sample all nestlings in under 3 minutes (Romero and Reed 2005). I collected blood from the first nestling sampled at each nest within less than 2 minutes on average of disturbing the nest. Third nestlings were sampled within about 14 minutes on average of disturbing the nest, and the amount of time between initial nest disturbance and blood collection was significantly different for first and third nestlings (t_{29}=-24.4, P<0.001). I used the first and third nestlings because the manipulated brood sizes ranged from 3 to 6 nestlings, and all nests had a first and third nestling to sample. I considered corticosterone levels in nestlings sampled first as baseline and

corticosterone levels in nestlings sampled third as stress-induced. I ran separate analyses for the first and third nestlings sampled. I created linear models with brood size group and date as fixed effects. I did not run separate analyses for male and female nestlings for corticosterone because I reduced my overall sample size by restricting analyses to first and third nestlings. Instead, I included sex as an additional fixed effect in the models. I also did not include nest ID as a random effect in these models because each model was run using data from only one nestling from each nest (either the first nestling sampled or third nestling sampled).

I used mixed models with brood size group and date as fixed effects, and manipulated nest ID and natal nest ID as random effects to determine the effect of brood size group on telomere lengths on days 2 and 15, and the rate of telomere loss (difference between telomere lengths on day 2 and day 15). I ran separate analyses for males and females.

Because many of the color measures were significantly correlated, I performed principal components analyses (PCA) on chest and rump feathers for nestling bluebirds. I performed a varimax rotation on the components. I used the eigenvalues to determine which components to retain. I again used mixed models with brood size group and date as fixed effects, and manipulated nest ID and natal nest ID as random effects to analyze the effect of brood size group on nestling coloration. Because male and female nestlings have visibly different plumage coloration at day 15, I ran separate analyses for each sex.

I also tested how well the manipulated nest in which nestlings were reared, or nest of origin explained variation in several measurements collected on day 15: mass, corticosterone levels, telomere lengths, and feather color. I created mixed models with

brood size group as the fixed effect and fitted with restricted maximum likelihood (REML) estimations. I varied the random effects structure, using either manipulated nest ID or natal nest ID as the random effect, keeping the fixed effect the same. I used likelihood ratio tests to compare these mixed models to a model with no random effect to determine which of the models best explained variation in telomere lengths. I ran separate models for males and females.

I ran mixed models to determine relationships among nestling feather coloration, telomere length, corticosterone levels, and mass controlling for brood size group and date, and with manipulated nest ID and natal nest ID as random effects. I used a Bonferroni correction to correct for multiple tests. For these models, the corrected P-value is reported.

RESULTS

Effects of brood size manipulation on parental behavior

Males raising enlarged broods tended to enter the nest box more during the two hour observation period than males raising reduced broods ($F_{2,24} = 2.68$, $P = 0.09$; Fig. 4a), but the effect was not statistically significant. Female nestling provisioning behavior was affected by experimental group. Females raising enlarged broods entered the nest box significantly more during the two hour observation period than females raising reduced broods ($F_{2,24} = 9.02$, $P = 0.002$; Fig. 4b). However, when I analyzed the number of nest visits per nestling over the two hour time period, there was no effect of brood size group (males: $F_{2,24} = 0.48$, $P = 0.63$; females: $F_{2,24} = 1.27$, $P = 0.30$; Fig 5). Thus, although adults raising experimentally enlarged broods made more nest visits overall,

there was no difference among brood size groups in terms of parental visits (presumably for food delivery) to each individual nestling. I also examined nest visits in relation to the deviation from the original brood size (i.e., the number of nestlings added or removed from the nest), and in relation to final brood size, both of which I treated as continuous variables. When the deviation from the original brood size was larger (i.e., when more nestlings were added to the nest), males entered the nest box significantly more often ($F_{1,24}$=5.74, P=0.03), but male visits per nestling were not related to the deviation from the original brood size ($F_{1,24}$=0.65, P=0.43). When the final brood size was larger, males did not enter the nest box more often ($F_{1,24}$=2.22, P=0.15), but males made more visits per nestling when final brood sizes were larger ($F_{1,24}$=6.04, P=0.02). Female bluebirds entered the nest box significantly more often when the deviation from the original brood size was larger ($F_{1,24}$=13.0, P=0.002), but female visits per nestling were not related to brood size deviation ($F_{1,24}$=0.28, P=0.61). Females entered the nest box significantly more often when the final brood size was larger ($F_{1,24}$=11.09, P<0.01), but female nest visits per nestling were not related to final brood size ($F_{1,24}$=2.54, P=0.13).

Males raising enlarged broods tended to respond more aggressively to the house sparrow intruder than males raising reduced broods ($F_{2,24}$ = 2.55, P = 0.07). Female aggressive behavior was not significantly affected by experimental group ($F_{2,24}$ = 0.06, P = 0.95). Male aggression score was not significantly related to the deviation from the original brood size ($F_{1,24}$=2.29, P=0.15) or final brood size ($F_{1,24}$=2.29, P=0.15). Female aggression score also was not significantly related to the deviation from the original brood size ($F_{1,24}$=0.09, P=0.77) or final brood size ($F_{1,24}$<0.01, P=0.96).

Effects of brood size manipulation on offspring traits

Brood size group did not affect male nestling mass, but females raised in enlarged broods were heavier on day 15 (Table 1). Brood size group did not affect male or female growth rates (Table 1). There was no relationship between male day 15 mass and deviation from the original brood size ($F_{1,26}$=1.11, P=0.30), when controlling for date ($F_{1,26}$=3.06, P=0.09), or final brood size ($F_{1,26}$=0.73, P=0.40), when controlling for date ($F_{1,26}$=3.62, P=0.07). There was no relationship between female day 15 mass and deviation from the original brood size ($F_{1,29}$=0.99, P=0.32), when controlling for date ($F_{1,29}$=13.61, P<0.01), or final brood size ($F_{1,29}$=2.52, P=0.12), when controlling for date ($F_{1,29}$=11.68, P<0.01). There also was no relationship between male growth rates and brood size deviation ($F_{1,26}$=0.75, P=0.39), when controlling for date ($F_{1,26}$=5.28, P=0.03), or final brood size ($F_{1,26}$=0.70, P=0.41), when controlling for date ($F_{1,26}$=4.84, P=0.04). There was no relationship between female growth rates and brood size deviation ($F_{1,29}$=0.53, P=0.47), when controlling for date ($F_{1,29}$=4.51, P=0.04), or final brood size ($F_{1,29}$=2.05, P=0.16), when controlling for date ($F_{1,29}$=5.08, P=0.03). Male and female nestlings did not differ in day 15 mass ($F_{1,28}$=1.32, P=0.27) or growth rates ($F_{1,28}$=0.01, P=0.91).

Because females raised in enlarged broods were heavier on day 15, but did not differ from other brood size groups in growth rate or day 2 mass, we tested to see if there was a significant difference between day 11 mass and day 15 mass within brood size groups. Females raised in enlarged broods had a non-significant tendency to be heavier on day 15 than day 11 (paired t-test, t_{36}=-1.93, P=0.06). Females raised in reduced broods were not significantly heavier on day 15 than on day 11 (paired t-test, t_{14}=-1.04, P=0.32),

and females raised in control broods were not significantly heavier on day 15 than on day 11 (paired t-test, t_{25}=-0.48, P=0.64).

For the first birds sampled for baseline corticosterone measurements, there was no effect of brood size group or sex on corticosterone levels (Table 1). For the third birds sampled for stress-induced corticosterone measurements, brood size group did not affect corticosterone levels (Table 1). Corticosterone levels did not differ between sexes (Table 1). Baseline corticosterone levels were not related to brood size deviation ($F_{1,27}$=0.04, P=0.84), when controlling for date ($F_{1,27}$=12.53, P<0.01) and sex ($F_{1,27}$<0.01, P=0.97), or final brood size ($F_{1,27}$=1.21, P=0.28), when controlling for date ($F_{1,27}$=11.22, P<0.01) and sex ($F_{1,27}$=0.11, P=0.74). Stress-induced corticosterone levels were not related to brood size deviation ($F_{1,25}$=2.27, P=0.14), when controlling for date ($F_{1,25}$=0.02, P=0.89) and sex ($F_{1,25}$=1.40, P=0.27), or final brood size ($F_{1,25}$=1.67, P=0.21), when controlling for date ($F_{1,25}$=0.23, P=0.64) and sex ($F_{1,25}$=1.32, P=0.29).

Nestling telomere lengths on day 2 did not differ across brood size groups for males ($F_{2,22}$=1.88, P=0.18) or females ($F_{2,24}$=0.58, P=0.56). Brood size group did not affect telomere length on day 15 or the rate of telomere loss for male or female nestlings (Table 1). Male telomere lengths at day 15 were not significantly related to brood size deviation ($F_{1,29}$=0.52, P=0.48), when controlling for date ($F_{1,29}$=0.05, P=0.83), or final brood size ($F_{1,29}$=0.81, P=0.38), when controlling for date ($F_{1,29}$<0.01, P=0.98). Male telomere loss was not significantly related to brood size deviation ($F_{1,29}$=0.28, P=0.60), when controlling for date ($F_{1,29}$=0.03, P=0.85), or final brood size ($F_{1,29}$=0.03, P=0.86), when controlling for date ($F_{1,29}$=0.06, P=0.81). Female day 15 telomere lengths also were not significantly related to brood size deviation ($F_{1,26}$=0.23, P=0.64), when controlling for

date ($F_{1,26}$=0.80, P=0.38), or final brood size ($F_{1,26}$=0.39, P=0.54), when controlling for date ($F_{1,26}$=0.70, P=0.41). Female telomere loss was not significantly related to brood size deviation ($F_{1,26}$=2.28, P=0.14), when controlling for date ($F_{1,26}$=2.65, P=0.11), or final brood size ($F_{1,26}$=0.48, P=0.49), when controlling for date ($F_{1,26}$=3.35, P=0.08).

Male and female nestling bluebirds differed significantly in their telomere lengths at day 15, as well as in their rate of telomere shortening. On day 15, males had significantly longer telomeres than females ($F_{2,27}$=5.21, P=0.007), when controlling for brood size group ($F_{2,27}$=1.63, P=0.20) and date ($F_{1,22}$=0.02, P=0.88). Males also experienced less telomere loss between day 2 and day 15 than females ($F_{2,27}$=4.82, P=0.01), when controlling for brood size group ($F_{2,27}$=0.80, P=0.45) and date ($F_{1,22}$=0.38, P=0.54). Day 2 telomere lengths did not differ between the sexes ($F_{2,27}$=0.14, P=0.87).

The results of the PCA are reported in Table 2. Brood size group had a significant effect on male rump PC1 scores. Compared to males raised in reduced broods, males raised in enlarged broods had rump feathers that were less ultraviolet and less violet saturated (Table 1). Males raised in enlarged broods tended to have less bright (i.e., darker) and more red saturated chest feathers than males raised in reduced broods; however, this relationship was not significant (Table 1). There was no significant effect of brood size group on rump PC2 scores (Table 1). I found that male chest PC1 was not significantly related to brood size deviation ($F_{1,26}$=0.08, P=0.78), when controlling for date ($F_{1,26}$=2.46, P=0.13), or final brood size ($F_{1,26}$=0.02, P=0.89), when controlling for date ($F_{1,26}$=2.40, P=0.13). Male rump PC1 was not significantly related to brood size deviation ($F_{1,26}$=1.99, P=0.17), when controlling for date ($F_{1,26}$=6.32, P=0.02), or final brood size ($F_{1,26}$=0.08, P=0.78), when controlling for date ($F_{1,26}$=4.66, P=0.04). Male

rump PC2 was not significantly related to brood size deviation ($F_{1,26}$=0.008, P=0.93), when controlling for date ($F_{1,26}$=0.01, P=0.92), or final brood size ($F_{1,26}$=0.05, P=0.83), when controlling for date ($F_{1,26}$<0.01, P=0.95).

Brood size group had a significant effect on female rump PC1 scores, and chest PC1 scores, and a non-significant effect on rump PC2 scores. Females raised in enlarged broods had rump feathers that were less ultraviolet and violet saturated, lighter, and lower in hue than females raised in reduced broods (Table 1). Females raised in enlarged broods had chest feathers that were darker and more red saturated than females raised in reduced broods (Table 1). Rump PC1 and PC2 scores were also significantly related to the deviation from the original brood size (rump PC1: $F_{1,26}$=11.56, P<0.01; rump PC2=7.51, P<0.01), when controlling for date (rump PC1: $F_{1,26}$=15.03, P<0.01; rump PC2: $F_{1,26}$=1.95, P=0.17), meaning that females raised in nests with the largest increases from the original brood size had less ultraviolet and violet saturated rump feathers that were lighter and lower in hue. Rump PC1 and PC2 scores were significantly related to final brood size (rump PC1: $F_{1,26}$=7.04, P=0.01; rump PC2=9.53, P<0.01), when controlling for date (rump PC1: $F_{1,26}$=10.90, P<0.01; rump PC2: $F_{1,26}$=1.63, P=0.21)Female chest PC1 scores were not significantly related to brood size deviation ($F_{1,26}$=0.91, P=0.35), when controlling for date ($F_{1,26}$=5.59, P=0.02), or final brood size ($F_{1,26}$=1.34, P=0.25), when controlling for date ($F_{1,26}$=5.88, P=0.02).

When comparing models with manipulated nest ID or natal nest ID as random effects to a model with no random effects, I found that both manipulated and natal nest explained variation in day 15 mass for male (Table 3) and female (Table 4) nestlings. Neither manipulated nor natal nest explained variation in male nestling corticosterone

levels (Table 5), but natal nest did explain variation in corticosterone levels in female nestlings (Table 6). Both manipulated and natal nest explained variation in telomere lengths for males (Table 7) and females (Table 8). For male nestlings, manipulated nest and natal nest did not explain variation in feather color (Table 9). For female nestlings, natal nest explained the variation in chest PC1, and manipulated nest explained the variation in rump PC1 and rump PC2.

Relationships among offspring traits

I did not find any relationships between male nestling rump or chest color and mass (rump PC1: $F_{1,26}$=0.07, corrected P=1.00; rump PC2: $F_{1,26}$=0.002, corrected P=1.00; chest PC1: $F_{1,26}$=0.97, corrected P=1.00). There were no relationships between female nestling rump or chest color and mass (rump PC1: $F_{1,29}$=3.04, corrected P=0.27; rump PC2: $F_{1,29}$=0.27, corrected P=1.00; chest PC1: $F_{1,29}$=1.39, corrected P=0.75).

There was no relationship between mass and telomere lengths for male or female nestlings on day 2 (males: $F_{1,27}$=0.32, corrected P=1.00; females: $F_{1,27}$=0.003, corrected P=1.00) or day 15 (males: $F_{1,27}$=0.99, corrected P=0.66; females: $F_{1,27}$=0.19, corrected P=1.00). There also was no relationship between day 15 mass and telomere loss for male or female nestlings (males: $F_{1,27}$=0.03, corrected P=1.00; females: $F_{1,27}$=2.83, corrected P=0.20). There was no relationship between growth rate and telomere lengths on day 15 for males ($F_{1,27}$=0.14, corrected P=1.00) or females ($F_{1,27}$=0.18, corrected P=1.00). There was no relationship between growth rate and telomere loss for males ($F_{1,27}$=1.41, corrected P=0.50) or females ($F_{1,27}$=0.01, corrected P=1.00).

Male rump PC2 scores were not significantly related to the rate of telomere loss, but there was a non-significant tendency for males with darker rump feathers to have slower rates of telomere loss ($F_{1,26}$=5.72, corrected P=0.09). I did not find a relationship between rump PC1 scores and rate of telomere loss ($F_{1,26}$=0.31,corrected P=1.00), nor did I find a relationship between chest coloration and rate of telomere loss ($F_{1,26}$=0.38, corrected P=1.00). Telomere lengths on day 15 were not related to rump coloration (rump PC1: $F_{1,26}$=0.07, corrected P=1.00; rump PC2: $F_{1,26}$=2.70, corrected P=0.36), or chest coloration ($F_{1,26}$=0.38, corrected P=1.00).

Female rump coloration was not related to day 15 telomere lengths (rump PC1: $F_{1,29}$=2.12, corrected P=0.45; rump PC2: $F_{1,29}$=0.48, corrected P=1.00). Female chest coloration was not related to day 15 telomere lengths ($F_{1,29}$=4.44, corrected P=0.12). Female rump coloration also was not related to rate of telomere loss (rump PC1: $F_{1,29}$=0.23, corrected P=1.00; rump PC2: $F_{1,29}$=0.63, corrected P=1.00), nor was chest coloration ($F_{1,29}$=0.97, corrected P=1.00).

Neither baseline nor stress-induced corticosterone levels were related to mass at fledging (all corrected P > 0.48). There were no relationships between either baseline or stress-induced corticosterone levels and telomere length at day 15 or rate of telomere loss for male or female nestlings (all corrected P > 0.50). There also were no relationships between either baseline or stress-induced corticosterone levels and any feather color measurements for male or female nestlings (all corrected P > 0.33).

DISCUSSION

In this study, I conducted a brood size manipulation using eastern bluebirds to test the hypothesis that being raised in a large brood carries costs for offspring due to reduced parental care. In general, my hypothesis was not supported. Overall, adults fed enlarged broods at higher rates, such that feeding rate per individual nestling did not differ across brood size groups. This compensation by parents for increased brood size was likely the reason I detected few costs of increased brood size on offspring. Male nestling mass was not affected by brood size group, but females raised in enlarged broods were heavier, which was the predicted relationship. Neither baseline nor stress-induced corticosterone levels were affected by the brood size manipulation. Telomere lengths and telomere loss were also not affected. However, feather coloration of both male and female nestlings was affected by brood size group. Also, independent of the brood size manipulation, I found that male and female nestlings differed in telomere loss during the nestling period and telomere lengths at fledging.

Lack (1947) proposed that clutch sizes in birds are adjusted based on how many offspring the parents are able to raise, but costs to parents of increasing brood size must also be accounted for (Monaghan and Nager 1997). In my study, adults raising experimentally enlarged broods provided more care overall than adults raising reduced broods. Female bluebirds raising enlarged broods made more nest box visits than females raising reduced broods. Similarly, males raising enlarged broods tended to visit the nest box more than males raising reduced broods. As a result, when I examined nest visit rates per nestling, there was no difference among brood size groups. Siefferman and Hill (2007a) conducted a brood size manipulation on eastern bluebirds, and found that parents

raising enlarged broods fed each nestling less than parents of reduced broods. This situation created "poor" and "good" rearing environments for nestlings (Siefferman and Hill 2007a). The adults raising enlarged broods in our population, on the other hand, appeared to compensate for the larger brood sizes by increasing feeding rates. Thus, nestlings in enlarged broods did not incur many costs (that I measured) associated with brood size, but adults of enlarged broods may have incurred costs instead. We might expect parents to bear the costs of increased brood size when the benefits of investing in current reproduction outweigh costs to parents and the ability to invest in reproduction in the future (Trivers 1972; Dijkstra et al. 1990; Clutton-Brock 1991; Stearns 1992). Consistent with these predictions, Ardia (2005) found differences in parental investment by female tree swallows (*Tachycineta bicolor*) breeding at different latitudes and with different life history strategies. Female tree swallows in Alaska adjusted parental care in response to a brood size manipulation so offspring quality did not differ among broods (Ardia 2005). In Tennessee, female tree swallows did not adjust parental investment so offspring in enlarged broods were of lower quality (Ardia 2005). Alaska females have lower return rates compared to Tennessee females, so Alaska females may have adopted a strategy that maximizes current reproductive payoffs over future reproductive payoffs (Ardia 2005). Another brood size manipulation study on eastern bluebirds found sex-specific costs of increasing parental care, which might be due to differences between males and females in reproductive investment strategies (Siefferman and Hill 2007b). Females raising enlarged broods were less likely to survive than males, suggesting that males might be less willing to increase care for enlarged broods at the cost of increased mortality because of paternity uncertainty (Siefferman and Hill 2007b). In our bluebirds,

both males and females might have been likely to bear the cost of raising an enlarged brood if success of the nest enhanced fitness in similar ways for both sexes. When I regressed nest visits against final brood size as a continuous variable, I found that females raising larger broods made more visits to the nest box, but this pattern did not hold for males. I do not have information about paternity for our population, but combining these data with nest visit data would inform us if certainty of paternity increases the likelihood that males incur similar costs to females.

It is important to note that the manipulated brood sizes in my study were within the natural range for our population. Siefferman and Hill (2007a) created manipulated brood sizes that also were within the natural range and similar to ours, but other environmental factors (e.g., food availability) may have prevented adults of enlarged broods from compensating in this previous study. Food availability can limit reproduction (Martin 1987), and at least one study on boreal owls (*Aegolius funereus*) has shown that parents will compensate for enlarged broods during years with high food availability, but not during low food availability years (Korpimäki 1988). The manipulated brood sizes in my study did not deviate very much from original brood sizes, so if food availability was high during the year when I manipulated brood sizes, parents might have been better able to increase effort for enlarged broods. To successfully create "poor" rearing conditions, especially in years with high natural food availability, it might be necessary to create enlarged broods that are much larger than the largest natural brood size of six nestlings. Such a manipulation would be less ecologically relevant, but may reveal selection pressures on clutch size. Alternatively, the ability of parents to compensate for large brood sizes could be manipulated to limit their feeding behavior.

The brood size manipulation did not affect nestling male mass, but it affected female mass in an unexpected way; female nestlings raised in enlarged broods were heavier at fledging. This pattern was not an effect of females being heavier to begin with because there were no differences in female mass across brood size groups at the start of the experiment. It also was not an effect of faster growth of female nestlings in enlarged broods because growth rates did not differ across brood size groups. This effect may have occurred from females in enlarged broods gaining weight between days 11 and 15, a pattern that did not appear to have happened in females raised in control or reduced broods. The parameter that sets the inflection point and growth rate of the Gompertz equation, b_1, may not have captured this increase in mass that occurred close to fledging. Begging behavior of females in enlarged broods may have stimulated the parents to feed more. In great tits, nestlings raised in enlarged broods increased begging behavior, despite equal feeding rates of the different brood size groups by parents (Neuenschwander et al. 2003). In some cases, begging behavior can influence food allocation by adults. In tree swallows, nestlings that begged first received a larger proportion of the food brought by the parents (Whittingham et al. 2003). It is possible female nestling bluebirds in enlarged broods begged first or more intensely as they neared fledging.

In my study, nestling corticosterone levels were not affected by brood size group, which could be due to the compensatory behavior of the parents. I expected that nestlings raised in enlarged broods would have higher corticosterone levels as this pattern has been found in other brood size manipulation studies on songbirds (e.g., barn swallows; Saino et al. 2003). Contrary to the findings of Saino et al. (2003), Lobato et al. (2008) found

that that brood size did not affect corticosterone metabolites in droppings from nestling blue tits (*Cyanistes caeruleus*). In the study on blue tits, two nestlings were moved between nests to create reduced, control, and enlarged broods with approximately 6, 8, and 10 nestlings, respectively (Lobato et al. 2008). However, blue tit clutch sizes range from 4-14 eggs, so parents may have been able to adjust their provisioning rates to match the needs of enlarged broods (Lobato et al. 2008). Like Lobato et al. (2008), the enlarged broods in my study did not necessarily create stressful conditions for nestlings, which might be why corticosterone levels did not differ among brood size groups. Reduced food intake is known to elevate corticosterone levels in young birds. In black-legged kittiwakes (*Rissa tridactyla*), chicks on a restricted diet had higher baseline and stress-induced corticosterone levels compared to chicks fed *ad libitum* (Kitaysky et al. 1999). In Florida scrub jays (*Aphelocoma coerulescens*), nestlings whose parents fed at lower rates had higher corticosterone levels (Rensel et al. 2010).

Because food stress can increase corticosterone levels (Kitaysky et al. 1999; Rensel et al. 2010), and corticosterone exposure accelerates telomere loss in young birds (Herborn et al. 2014; Quirici et al. 2016), I expected that nestlings in enlarged broods would have shorter telomeres and greater telomere loss. Contrary to these predictions, the brood size manipulation did not affect nestling telomere lengths at fledging or the change in telomere lengths from day 2 to day 15. It was surprising that my results were not similar to those in Boonekamp et al. (2014), who found that nestling jackdaws raised in enlarged broods experienced greater telomere loss than nestlings in reduced broods. Again, parental compensation for enlarged broods likely decreased nestling hunger and competition among siblings, leading to similar telomere lengths across brood size groups.

My results were more similar to those in a study by Voillemot et al. (2012), in which there was no effect of brood size manipulation on nestling collared flycatcher (*Ficedula albicollis*) telomere lengths. In that study, nestlings were cross-fostered among three age-matched broods (brood triplets) to create enlarged, reduced, and control broods (Voillemot et al. 2012). Brood triplet and factors associated with triplets, such as location and hatch date explained variation in telomere lengths better than brood size group (Voillemot et al. 2012). I found that rearing environment (manipulated nest ID) and natal nest ID explained variation in telomere lengths for male and female nestlings. Moreover, rearing and natal environment explained variation in male and female day 15 mass and female feather coloration, and natal environment explained variation in female corticosterone levels. These results imply that pre-manipulation conditions (e.g., such as maternal effects or the hatching environment) as well as conditions associated with the rearing environment may impact telomere lengths, as well as other offspring traits in concert. More research using cross-fostering is needed to identify these factors and to tease apart the effects of natal versus rearing environments.

Male and female nestlings had different telomere loss rates and different telomere lengths, with males having slower telomere loss and longer telomeres at fledging. In some adult animals, including humans and rats, males have shorter telomeres and faster telomere loss than females, but in some bird species, including some that are sexually dimorphic, the sexes are equal in telomere lengths (reviewed in Barrett and Richardson 2011). Relatively few studies have compared sex differences in telomere loss and telomere lengths in young animals (Barrett and Richardson 2011). Many of the studies that have made this comparison show the sexes to be equal in telomere lengths at a young

age (Barrett and Richardson 2011). However, two studies found that young male birds had longer telomeres than females (European shags, *Phalacrocorax aristotelis*, Foote 2008; lesser black-backed gull, *Larus fuscus*, Foote et al. 2011a), and one study found that young female birds had longer telomeres than males (southern giant petrel, *Macronectus gigantus*, Foote et al. 2011b). Another study in European shags did not detect a sex difference in juveniles (Hall et al. 2004), but this study had a wider range of ages, suggesting that the sex difference may be present only when chicks are very young (Foote 2008). The sex difference in telomere lengths in the southern giant petrel might be related to differences in growth rates (Foote et al. 2011b). Male giant petrels grow faster than females, which could lead to shorter telomeres (Foote et al. 2011b). In the lesser black-backed gull, males are larger than females, but they also have longer telomeres, so differences in growth probably do not account for differences in telomere length (Foote et al. 2011a). In my study, males and females did not differ in mass or growth rates. In addition, telomere loss and telomere lengths were not related to mass in males or females. I thought these measurements might be correlated because mass at fledging is a good predictor of survival in birds (Both et al. 1999; Naef-Daenzer et al. 2001; Wheelright et al. 2003), and early life telomere lengths are related to survival (Heidinger et al. 2012). Given that the nestling bluebirds in my study were sampled only within the first 15 days of life, it is possible that additional telomere loss during the post-fledging stage might later result in similarity in telomere lengths between the sexes. Male and female nestlings did not differ in day 2 telomere lengths, so it also is possible that females are more sensitive to stressors that erode telomeres during the nestling stage, but males are more sensitive after fledging. Indeed, there is evidence that female offspring of other bird

species may be more sensitive to postnatal stress than males, exhibiting greater stress responses to corticosterone treatment, and increased mortality in relation to brood size manipulation (De Kogel 1997; Marasco et al. 2012; but see also Spencer and Verhulst 2007).

Brood size group affected feather color in male and female nestling bluebirds. Nestlings of both sexes raised in experimentally enlarged broods had less violet and ultraviolet saturated rump feathers. Siefferman and Hill (2007a) found that male bluebird nestlings raised in enlarged broods had less ornamented wing feathers and were fed less than nestlings raised in reduced broods, suggesting that food delivered to nestlings may play a role in the development of structural colors. Both male and female nestlings raised in enlarged broods in our population experienced adverse effects on rump ornamentation, despite no apparent differences among brood size groups in nest visits per nestling. It is possible that there was more competition among nestlings for food. Food quality may also impact feather quality. In this study, I only quantified visit rate, but not size or protein content of delivered food. Protein is important for the development of structural colors (Shawkey et al. 2003). Male nestling blue tits have a negative relationship between protein in blood plasma and UV chroma of the blue tail feathers, suggesting that nestlings that extract more protein from the blood are able to grow more ornamented feathers (Peters et al. 2007).

Siefferman and Hill (2007a) found that the negative impact of brood size on wing feather coloration was only present in male nestling bluebirds. Similarly, male, but not female, blue tit nestlings raised in enlarged broods had less ornamented tails than males raised in reduced broods (Jacot and Kempenaers 2007). Unlike these two studies, both

sexes in my study experienced the impact of brood size group on rump feather coloration. The UV blue feathers are thought to be sexually selected (Siefferman and Hill 2007a), so males might be expected to be more strongly affected by the adverse effects of brood size on their blue feathers. However, most body feathers, including those on the rump and chest, as well as 3–10 inner greater coverts, 1–3 tertial feathers, and 0 to all 12 tail feathers are not retained into the first breeding season, while the primary feathers are (Gowaty and Plissner 2015). Thus, the rump feathers grown as nestlings are probably not related to mating success in adulthood, which may be why both sexes were affected in my study. Instead, these feathers might convey important information to the parents when the young leave the dark nest box for the sun-lit areas outside. Indeed, adult bluebirds preferentially fed artificially brightened sons early in the season when those fledglings were heavier (Ligon and Hill 2010). Preferential feeding is likely to be especially important early in the season when food is more likely to be limited.

Brood size group affected nestling chest coloration as well. Females raised in enlarged broods had darker, more red saturated chest feathers than females raised in reduced broods. Male nestlings showed a similar pattern, though the effect on chest feathers was not as strong. The different effects of brood size on the different feather areas could be due to the fact that the colors of each feather area have different sources. The UV coloration of rump feathers is due to the nanostructure of the feather itself (Shawkey et al. 2003), but the red color on the chest is due to the melanin (specifically eumelanin and phaeomelanin) present in those feathers (McGraw et al. 2004). The effects on nestling chest coloration might be related to quantity or quality of food consumed, but there is conflicting evidence to support a direct relationship between nutrition and

melanin-based plumage color (e.g. McGraw et al. 2002), and in any case, female chest feather coloration was not related to mass at fledging.

It is surprising that feather coloration was impacted by the brood size manipulation even though many of the other measurements were not. It has been proposed that corticosterone is associated with production of melanin, possibly because of pleiotropic effects of melanin-stimulating hormone (reviewed in Ducrest et al. 2008). The proopiomelanocortin (POMC) gene produces melanin-stimulating hormone, which binds to melanocortin receptors (Ducrest et al. 2008). This binding action results in a number of effects, including initiation of melanin production and modulation of the stress response (Racca et al. 2005; Ducrest et al. 2008). In line with the suggestion that corticosterone levels and melanin-based plumage should be related, male barn swallows with darker feathers had higher baseline corticosterone levels and lower corticosterone responsiveness to an acute stressor, though this pattern only held for males with a reduced parental workload (Saino et al. 2013). Corticosterone levels are also related to the blue structural color of rump and tail feathers in adult bluebirds in our population (Grindstaff et al. 2012). In some birds, hatch order can have an effect on corticosterone levels. For example, American kestrel (*Falco sparverius*) chicks that hatched first had higher baseline corticosterone levels compared to chicks that hatched later (Love et al. 2003). However, in a study on eastern bluebirds, hatching order did not affect corticosterone levels (Soley et al. 2011). In my study, baseline and stress-induced corticosterone levels were not affected by brood size group, and were not related to any of the feather coloration measurements I took. Therefore, I cannot conclude that corticosterone was responsible for the effects of brood size group on nestling feather

coloration. However, I only measured one component of the HPA axis, but other components, such as the ability to return to baseline corticosterone levels after stress exposure (Almasi et al. 2010) should be considered. I also only measured corticosterone levels at one time point, but these measurements may not necessarily be an accurate reflection of an individual's corticosterone levels across a longer timescale (Jenkins et al. 2013).

To my knowledge, the relationships between telomere length and feather color in nestling birds have not previously been explored. After correcting for multiple tests in my study, I found that nestling male and female feather color was not significantly related to telomere length and telomere loss. In nestling barn swallows *(Hirundo rustica*), nestlings with faster feather growth had longer telomeres at fledging (Parolini et al. 2015), and in the turquoise-browed motmot (*Eumomota superciliosa*), male tail brightness was positively associated with faster tail feather growth (Murphy and Pham 2012). Thus, it is possible that with a larger sample size, meaningful relationships between telomeres and plumage ornamentation can be detected.

Early life stress can have long-term, negative impacts. For instance, most telomere loss probably occurs early in life (Hall et al. 2004; Pauliny et al. 2006), and early life telomere loss is related to lifespan (Heidinger et al. 2012; Boonekamp et al. 2014). Consequently, it would be worthwhile to compare telomere lengths across sexes in juvenile organisms. While males of some animals, particularly mammals, have shorter telomeres later in life, there are relatively few studies that show sexual differences in telomere dynamics early in life (Barrett and Richardson 2011). Sexual ornaments may also be particularly sensitive to early life stress (Siefferman and Hill 2007a). Here,

feather color was related to telomeres, even though telomeres were not affected by the brood size manipulation. Identifying additional sources of stress that may influence feather color will help us to understand how early life stress may affect ornamentation and the potential consequences of altered ornamentation.

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Table 1. Effect of brood size manipulation on nestling eastern bluebird feather color, telomere length, telomere loss, corticosterone levels, and mass. Feather color, telomere lengths, and mass results are from mixed models controlling for date, with manipulated and natal nests as random effects. Corticosterone results are from linear models controlling for date and sex.

Response variable	Predictors	Estimate	SE	F	df	P
Male day 15 mass	Brood size group[a]	1.18	1.59	0.43	2,26	0.66
	Date	0.04	0.02	4.14	1,26	0.05
Female day 15 mass	Brood size group[a]	0.81	0.74	3.50	2,29	**0.04**
	Date	-0.03	0.01	10.7	1,29	**0.002**
Male growth rate	Brood size group[a]	-0.01	0.03	0.40	2,26	0.67
	Date	-0.0008	0.0004	4.88	1,26	**0.03**
Female growth rate	Brood size group[a]	-0.03	0.03	0.08	2,29	0.92
	Date	-0.0008	0.0004	5.70	1,29	**0.02**
Baseline corticosterone	Brood size group[a]	0.17	0.67	1.78	2,27	0.19
	Date	-0.03	0.01	6.86	1,27	**0.01**
	Sex[b]	-0.002	0.54	<0.001	1,27	0.99

[a]Estimates are relative to reduced broods.

[b]Estimates are relative to males.

Table 1. Continued.

Response variable	Predictors	Estimate	SE	F	df	P
Stress-induced corticosterone	Brood size group[a]	-0.54	0.33	1.28	2,25	0.30
	Date	0.002	0.006	0.06	1,25	0.82
	Sex[b]	-1.45	0.81	1.59	1,25	0.22
Male day 15 telomere length	Brood size group[a]	0.33	0.86	0.54	2,29	0.59
	Date	0.003	0.004	0.05	1,29	0.82
Male telomere loss	Brood size group[a]	-0.31	0.99	0.52	2,29	0.60
	Date	-0.002	0.005	0.16	1,29	0.70
Female day 15 telomere length	Brood size group[a]	0.22	0.58	0.77	2,26	0.47
	Date	0.00	0.003	1.01	1,26	0.32
Female telomere loss	Brood size group[a]	-0.77	1.03	1.72	2,26	0.19
	Date	-0.002	0.005	2.10	1,26	0.16
Male Chest PC1	Brood size group[a]	2.23	11.5	2.77	2,26	0.08
	Date	0.04	0.07	1.18	1,26	0.29
Male Rump PC1	Brood size group[a]	36.7	15.4	4.63	2,26	**0.02**
	Date	0.23	0.09	3.10	1,26	0.09

[a]Estimates are relative to reduced broods.

[b]Estimates are relative to males.

Table 1. Continued.

Response variable	Predictors	Estimate	SE	F	df	P
Male Rump PC2	Brood size group[a]	-17.4	9.36	0.32	2,26	0.73
	Date	-0.10	0.05	0.03	1,26	0.88
Female Chest PC1	Brood size group[a]	20.1	8.94	4.26	2,26	**0.02**
	Date	0.14	0.05	6.18	1,26	**0.01**
Female Rump PC1	Brood size group[a]	7.39	16.1	5.72	2,26	**0.007**
	Date	0.08	0.09	11.0	1,26	**0.002**
Female Rump PC2	Brood size group[a]	6.33	9.40	3.19	2,26	0.05
	Date	0.02	0.05	1.96	1,26	0.17

[a]Estimates are relative to reduced broods.

[b]Estimates are relative to males.

Table 2. Eigenvectors for first and second principal components (PC) from a principal components analysis (PCA) of brightness, saturation, and hue of rump and breast feathers of nestling male and female eastern bluebirds.

Feather patch and color character	Male PC1	Male PC2	Female PC1	Female PC2
Rump (variance explained) (%)	(62)	(23)	(63)	(24)
Brightness	0.16	-0.81	0.39	0.54
Blue saturation	0.46	0.34	0.42	-0.42
Violet saturation	0.55	0.13	0.53	-0.23
Ultraviolet saturation	0.55	0.14	0.53	-0.24
Hue	-0.39	0.44	-0.34	-0.65
Breast (variance explained) (%)	(87)		(78)	
Brightness	0.71		0.71	
Red saturation	-0.71		-0.71	

Table 3. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 mass in male nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	-101.22	4			
2	Manipulated nest[b]	-99.13	5	4.17	1 vs. 2	**0.02**
3	Natal nest[c]	-98.92	5	4.57	1 vs. 3	**0.02**

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched

Table 4. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 mass in female nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	-170.08	4			
2	Manipulated nest[b]	-167.62	5	4.93	1 vs. 2	**0.01**
3	Natal nest[c]	-165.08	5	10.00	1 vs. 3	**<0.01**

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched

Table 5. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 baseline corticosterone levels in nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	-101.22	4			
2	Manipulated nest[b]	-100.22	5	1.98	1 vs. 2	0.08
3	Natal nest[c]	-101.13	5	0.17	1 vs. 3	0.34

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched

Table 6. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 stress-induced corticosterone levels in nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	-124.48	4			
2	Manipulated nest[b]	-125.19	5	2.57	1 vs. 2	0.05
3	Natal nest[c]	-124.84	5	3.27	1 vs. 3	**0.04**

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched

Table 7. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 telomere lengths in male nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	-9.05	4			
2	Manipulated nest[b]	-7.32	5	3.45	1 vs. 2	**0.03**
3	Natal nest[c]	-7.27	5	3.54	1 vs. 3	**0.03**

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched

Table 8. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 telomere lengths in female nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
1	No random effect	6.52	4			
2	Manipulated nest[b]	8.32	5	3.60	1 vs. 2	**0.03**
3	Natal nest[c]	11.0	5	8.93	1 vs. 3	**0.001**

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched

Table 9. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 feather color in male nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
Chest PC1						
1	No random effect	-73.40	4			
2	Manipulated nest[b]	-72.34	5	2.14	1 vs. 2	0.07
3	Natal nest[c]	-72.56	5	1.69	1 vs. 3	0.10
Rump PC1						
1	No random effect	-73.40	4			
2	Manipulated nest[b]	-72.34	5	2.14	1 vs. 2	0.07
3	Natal nest[c]	-72.56	5	1.69	1 vs. 3	0.10
Rump PC2						
1	No random effect	-73.40	4			
2	Manipulated nest[b]	-72.34	5	2.14	1 vs. 2	0.07
3	Natal nest[c]	-72.56	5	1.69	1 vs. 3	0.10

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched

Table 10. Results of likelihood ratio test to determine how well manipulated nest and nest of origin explain the variation in day 15 feather color in female nestling bluebirds. Models were fitted with REML with brood size group as a fixed effect, and models 2 and 3 included either manipulated nest ID or natal nest ID as a random effect.

Model	Model random structure	LL[a]	DF	Likelihood ratio (χ^2)	Test	P
Chest PC1						
1	No random effect	-89.64	4			
2	Manipulated nest[b]	-89.44	5	0.41	1 vs. 2	0.26
3	Natal nest[c]	-85.17	5	8.95	1 vs. 3	**<0.01**
Rump PC1						
1	No random effect	-115.67	4			
2	Manipulated nest[b]	-113.27	5	4.81	1 vs. 2	**0.01**
3	Natal nest[c]	-114.67	5	2.0	1 vs. 3	0.08
Rump PC2						
1	No random effect	-115.67	4			
2	Manipulated nest[b]	-113.27	5	4.81	1 vs. 2	**0.01**
3	Natal nest[c]	-114.67	5	2.0	1 vs. 3	0.08

[a]LL=log likelihood of models fitted with restricted maximum likelihood estimations.

[b]Manipulated nest is the rearing environment.

[c]Natal nest is the nest in which young were hatched.

Figure 1. Gompertz nestling growth curves showing the effect of varying a_0, the parameter that sets the asymptote, while keeping b_0, the parameter that sets the intercept, and b_1, the parameter that sets the growth rate and inflection point fixed. Curves were modeled from separate fits of the Gompertz function using individual nestling mass.

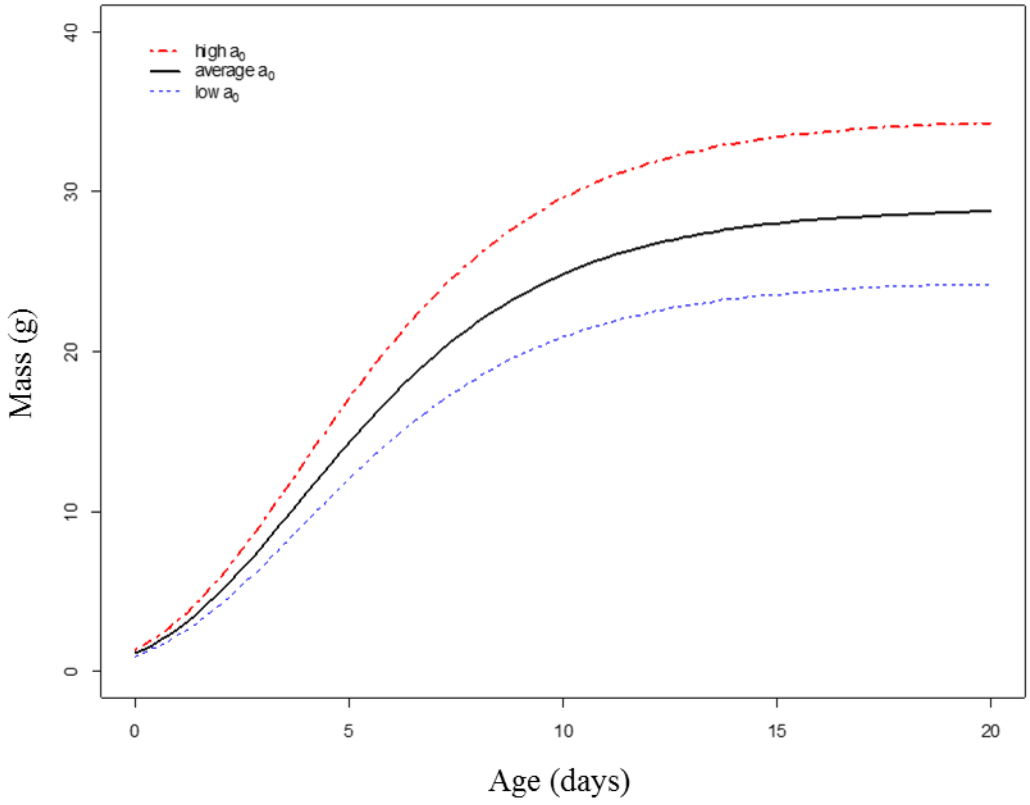


Figure 2. Gompertz nestling growth curves showing the effect of varying b_0, the parameter that sets the intercept, while keeping a_0, the parameter that sets the asymptote, and b_1, the parameter that sets the growth rate and inflection point, fixed. Curves were modeled from separate fits of the Gompertz function using nestling mass.

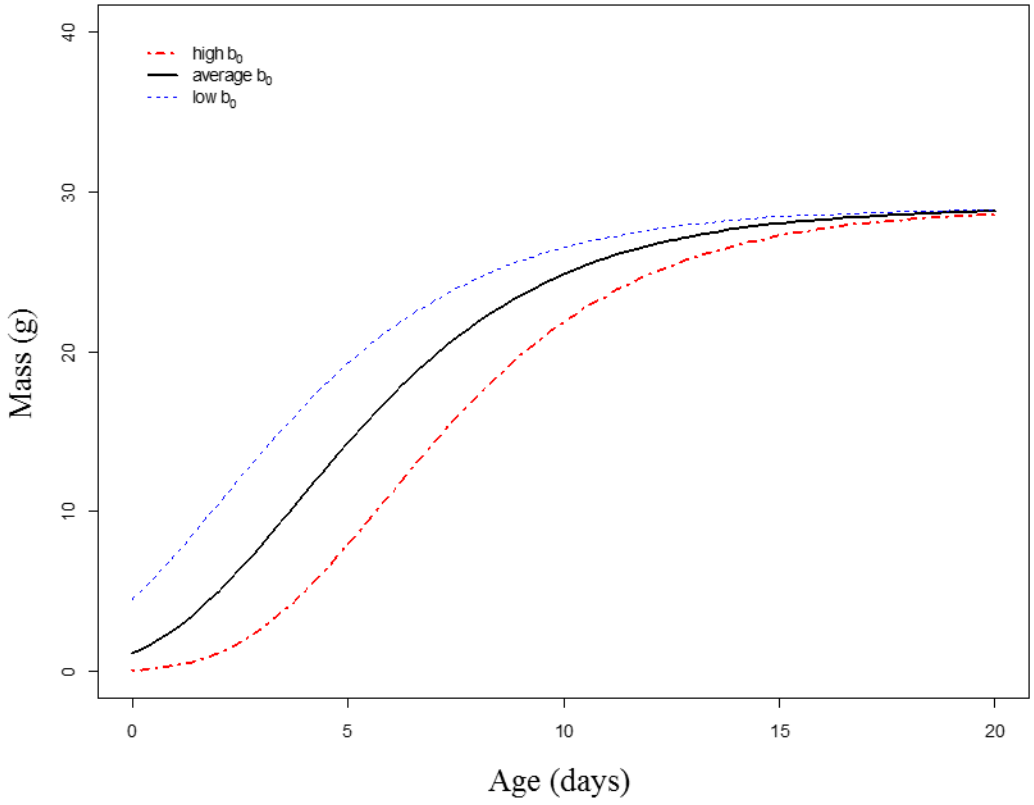


Figure 3. Gompertz nestling growth curves showing the effect of varying b_1, the parameter that sets the growth rate and inflection point, while keeping a_0, the parameter that sets the asymptote, and b_0, the parameter that sets the intercept, fixed. Curves were modeled from separate fits of the Gompertz function using nestling mass.

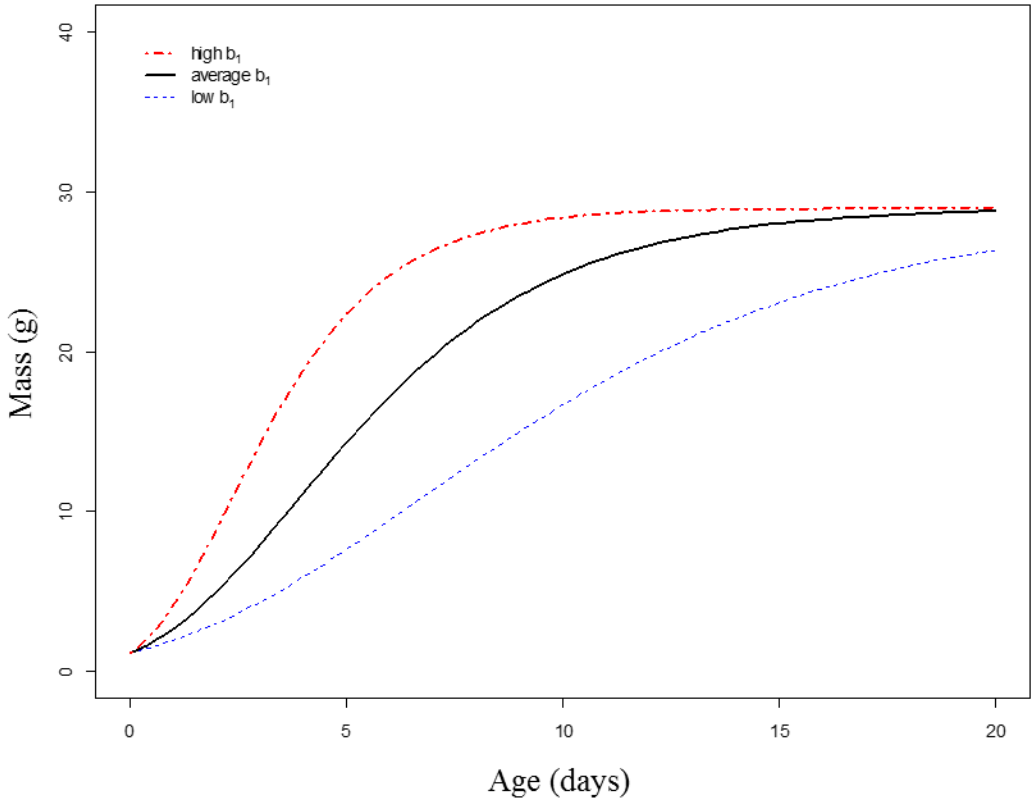


Figure 4. Nest box visits for a) males and b) females. Dark gray bars represent enlarged broods, black bars represent control broods, and light gray bars represent reduced broods. Males raising enlarged broods tended to visit the nest box more often than males raising reduced broods. Females raising enlarged broods made significantly more nest visits than females raising reduced broods.

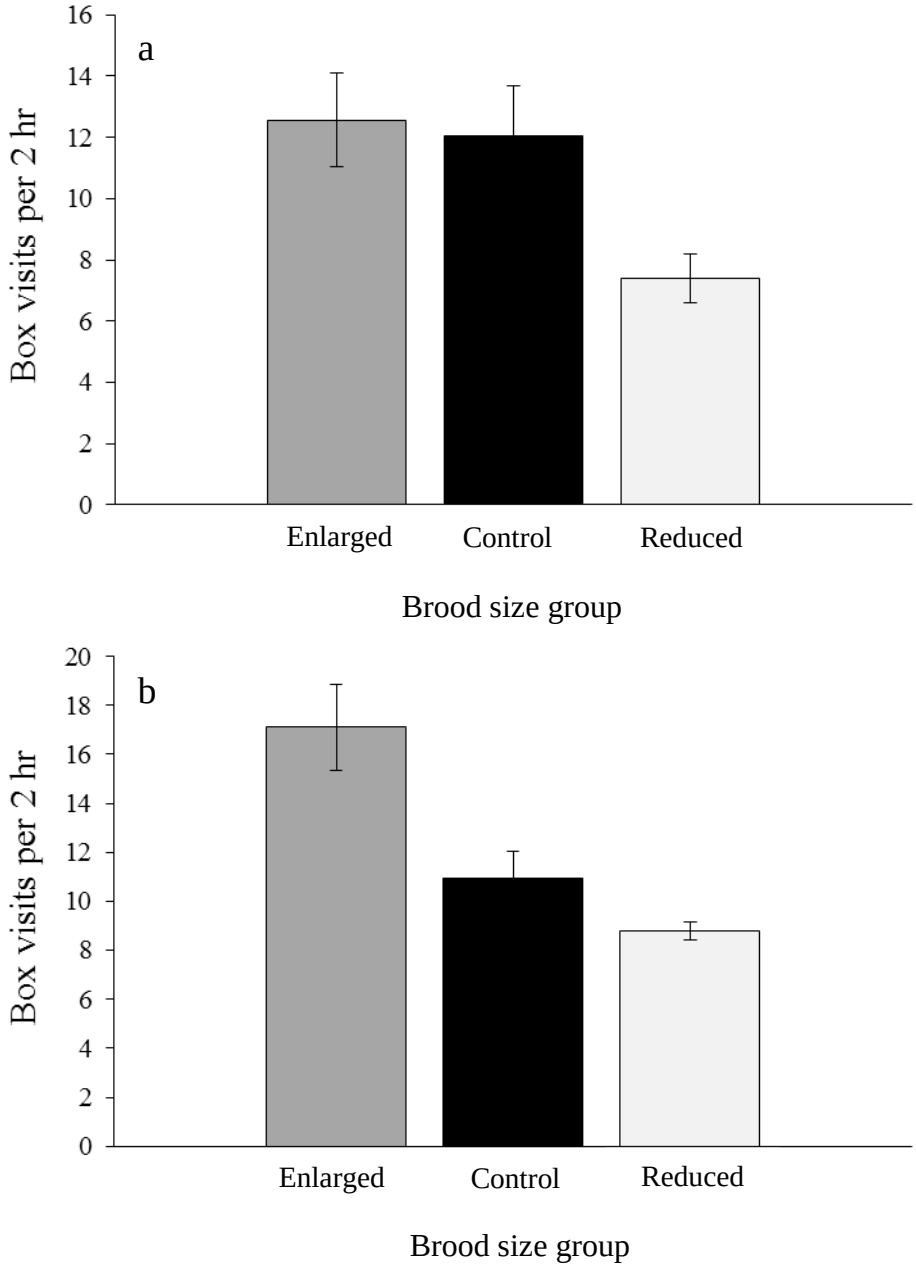


Figure 5. Nest box visits per individual nestling for a) males and b) females. Dark gray bars represent enlarged broods, black bars represent control broods, and light gray bars represent reduced broods. Feeding rates per nestling per 2 hours did not differ across brood size groups for males or females.

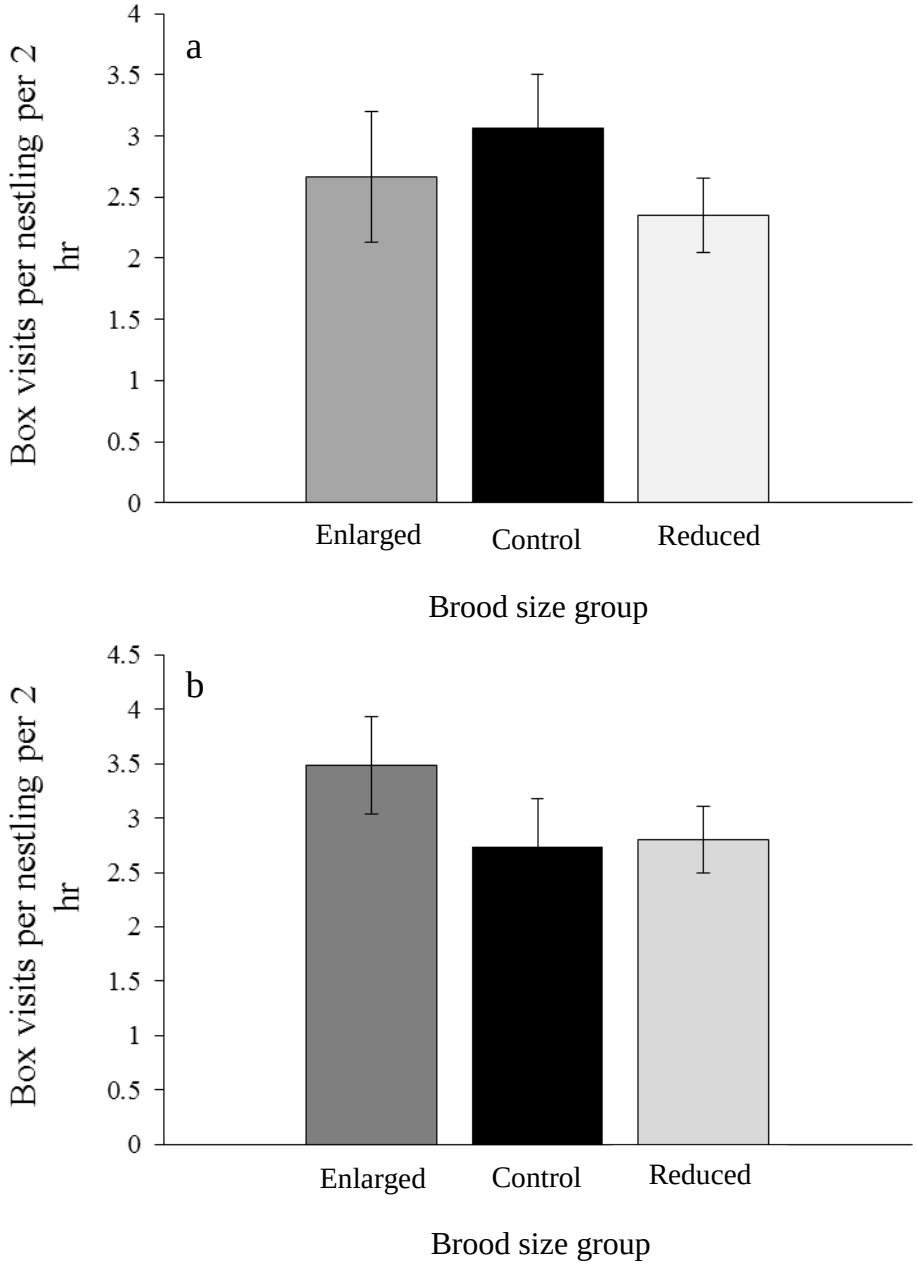
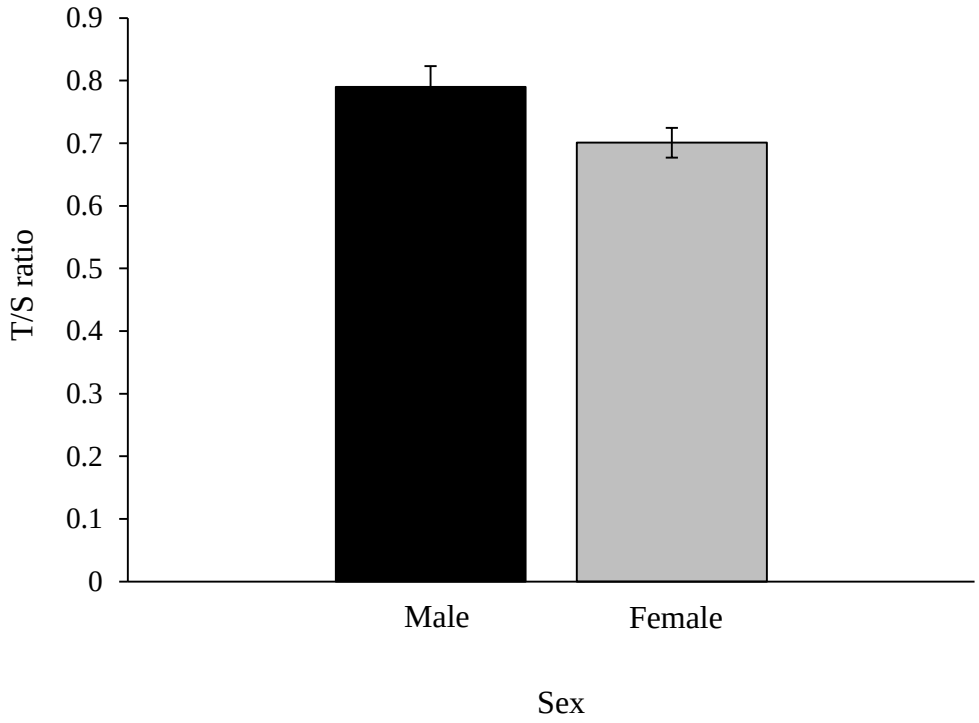


Figure 6. Differences in male and female nestling telomere lengths on day 15. Male nestling bluebirds had significantly longer telomere lengths on day 15 than female nestlings.



CHAPTER V

SUMMARY

SUMMARY

In my dissertation, I sought to address several objectives within three different chapters. In Chapter II, I characterized variation and repeatability of testosterone levels within individual eastern bluebirds (*Sialia sialis*), and assessed relationships between parental and aggressive behaviors and gonadotropin-releasing hormone- (GnRH-) induced testosterone levels and testosterone production. In chapter III, I tested for relationships between fitness measurements and initial (pre-GnRH) testosterone levels and GnRH-induced testosterone levels in male and female bluebirds. In Chapter IV, I determined how a brood size manipulation affected bluebird adult parental behavior and assessed potential costs to offspring in relation to their rearing environment.

When I examined testosterone levels of individuals, I found that initial testosterone levels varied significantly among individuals. I also found that, while males on average increased testosterone levels in response to GnRH, at the individual level some males decreased testosterone levels. Females on average did not respond to GnRH, but at the individual level, they too varied in their response. Male initial testosterone

levels, and male and female GnRH-induced testosterone levels were also individually repeatable across parental and aggressive behavioral contexts. When repeatability of a trait is observed, the trait might have a genetic basis, suggesting that it could be heritable (Lessells and Boag 1987). Future studies would benefit by conducting cross-fostering experiments and testing the response to GnRH in adult offspring in relation to those of foster or genetic parents.

I did not detect relationships between provisioning rates and initial testosterone levels, GnRH-induced testosterone levels, or testosterone production in either males or females. This pattern was surprising because considerable previous studies on both sexes have found a negative relationships between testosterone and parental care (e.g., Wingfield et al. 1990; O'Neal et al. 2008). I hypothesized that males in our population may have become "behaviorally insensitive" to testosterone (Lynn et al. 2002; Lynn 2008; Lynn 2016). This situation is expected to occur in male birds when paternal care is essential to success of the nest and the suppressive effects of testosterone on parental care might interfere with important behaviors (Lynn et al. 2002; Lynn 2008; Lynn 2016). The relationship between female parental behavior and testosterone as well as the response to GnRH might vary over the nesting cycle (Jawor et al. 2007). It is possible that if I had sampled females during egg laying or incubation, I might have found the predicted negative relationship between testosterone and parental behavior. It is also possible that other hormones that have been implicated in parental care, such as prolactin (Wingfield and Goldsmith 1990), might have had a stronger influence on provisioning behavior than testosterone in our bluebird population.

Testosterone levels were not related to reproductive success in male and female bluebirds. Thus, I did not find evidence that selection might be acting on testosterone levels in our population. These findings contrasted those from McGlothlin et al. (2010), which demonstrated positive directional selection on GnRH-induced testosterone levels in male dark-eyed juncos (*Junco hyemalis*). Future research that builds from these two studies should quantify within- and extra-pair mating success for males and females for a more robust proxy for reproductive success.

While parental care was not related to testosterone, I still found it to be important in shaping the offspring rearing environment. Siefferman and Hill (2007) found that brood size reduced provisioning rates, creating "poor" and "good" rearing environments for offspring. In our population, adult bluebirds raising enlarged broods increased provisioning rates, effectively compensating for the larger brood sizes. Thus, offspring did not incur many costs based on what I measured. Instead, adults may have experienced the cost of investing more in larger broods. Also, the manipulated brood sizes were not largely different from the original sizes and were still within the natural range, potentially making it easier for adults raising enlarged broods to compensate. Interestingly, while growth, corticosterone levels, and telomere lengths were not affected by the brood size manipulation, chest and rump feather coloration in male and female nestlings were impacted. I did not measure quality of food delivered to offspring, which could influence some aspects of feather coloration (Peters et al. 2007). In addition, other components of the stress axis that I did not measure, or corticosterone levels on a larger timescale could potentially influence feather coloration (Almasi et al. 2010; Jenkins et al. 2013).

My research complements studies in which hormone levels are manipulated by presenting natural variation in hormone production capabilities. The field would likely benefit from studies that measure hormone levels at multiple points across time or contexts, and determine how those levels predict fitness-related traits. In addition, the results from my brood size manipulation experiment highlight the need to gain additional understanding of when adults will be more likely to bear costs of increased investment, and how their responses influence the early life experiences of their young.

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VITA

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Thesis: INDIVIDUAL VARIATION IN TESTOSTERONE LEVELS AND PARENTAL CARE IN EASTERN BLUEBIRDS (*SIALIA SIALIS*): INTERACTIONS WITH REPRODUCTIVE SUCCESS AND OFFSPRING

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