THE ROLE OF MATERNAL HORMONES IN PROGRAMMING OFFSPRING AGGRESSION

by

ALEXANDRA B. BENTZ

(Under the Direction of Kristen J. Navara)

ABSTRACT

Over the past few decades, researchers have come to recognize that a mother's physiological response to her environment can have transgenerational effects. Females transfer testosterone to their young prenatally and this maternal effect makes offspring more aggressive. Aggression can strongly influence an animal's success depending on environmental context, and maternal effects are an important source of variation in aggressive behavior; yet, we do not fully understand what shapes maternal hormone responses or the proximate mechanisms that mediate its effects. While it is generally assumed that females in competitive environments allocate more testosterone, adaptively creating more aggressive offspring, not all bird species (in which maternal effects are best studied) respond by allocating more testosterone to egg yolks, making it hard to predict how different bird species' behaviors and egg components will respond to environmental change. Additionally, we do not know what mechanisms mediate the effects of prenatal testosterone on offspring behavior, preventing us from understanding how it fits into larger ecological and evolutionary frameworks. Hence, my dissertation addressed 1) the causes of interspecies variation in yolk testosterone allocation in competitive environments and 2) the molecular mechanisms facilitating behavioral plasticity in offspring exposed to prenatal

testosterone. For Aim 1, I performed a meta-analysis to identify species-specific traits influencing yolk testosterone allocation (Chapter 2) and experimentally tested the findings (Chapter 3). I found that colonial species do not allocate more yolk testosterone in competitive environments, unlike solitary species. This work challenges a widely held assumption that this maternal effect is characterized by a uniform response to competition, showing that it should be contextualized with life-history traits. Next, I explored natural variation in molecular responses to yolk testosterone in a wild songbird (Chapter 4) and experimentally tested the mechanisms in a captive species (Chapter 5). Hundreds of neural genes are differentially expressed in offspring exposed to yolk testosterone, including genes in behavioral pathways (e.g., nitric oxide and serotonin). Additionally, the data suggest epigenetic mechanisms may play a role in mediating the effects of maternal testosterone on offspring phenotype. Ultimately, this work helps us better understand the environmental and molecular causes of phenotypic plasticity.

INDEX WORDS: yolk testosterone, aggressive behavior, avian, maternal effect, competitive environment, DNA methylation

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Overview

Females transfer steroid hormones to their offspring during prenatal development and the concentration transferred is directly related to physical and social factors in their environment. Maternal effects such as this are common across taxa (hyaenas, Dloniak et al. 2006; guinea pigs, Kemme et al. 2007; baboons, Onyango et al. 2008; trout, Burton et al. 2011; squirrels, Dantzer et al. 2011), but are best studied in birds, because their young develop externally in eggs and there is substantial variation in egg yolk hormones (Groothuis et al. 2005). One of the most commonly studied maternal effects in birds is the transfer of maternal testosterone to egg yolks as a consequence of maternal social environment. Female birds breeding in competitive environments, such as high breeding density, transfer more testosterone to their egg yolks (Schwabl 1997; Reed and Vleck 2001; Whittingham and Schwabl 2002; Mazuc et al. 2003; Pilz and Smith 2004; Navara et al. 2006a; Eising et al. 2008; Hargitai et al. 2009; Guibert et al. 2010; Remeš 2011; Bentz et al. 2013). Increased yolk testosterone generally increases nestling growth (Schwabl 1996a; Lipar and Ketterson 2000; Navara et al. 2005; Navara et al. 2006a; Müller et al. 2008), metabolic rate (Tobler et al. 2007; Nilsson et al. 2011), nestling competitive ability (i.e., increased begging rate and vigor during nestling food competition; Schwabl 1996a; Eising and Groothuis 2003; Bentz et al. 2013), and aggression as adults (Strasser and Schwabl 2004; Eising et al. 2006; von Engelhardt et al. 2006; Partecke and Schwabl 2008; Müller et al. 2009).

Many researchers have postulated that maternal effects cause adaptive phenotypic plasticity, because they generate seemingly beneficial phenotypes for the prevailing environment (Pilz et al. 2004; Storm and Lima 2010; Meylan and Clobert 2005; Dantzer et al. 2013). Maternal-derived testosterone, for example, increases offspring aggression in response to competitive environments (see above). Natural selection tends to favor aggressive individuals in competitive environments (Biro and Stamps 2008; Rosvall 2008), but not in other environments, like predator-dense environments (Biro et al. 2004), as aggressive individuals also take more risks (Sih et al. 2004; Reaney and Backwell 2007). Animal behaviors, like aggression, are consistent across contexts (Sih et al. 2004; Bell et al. 2009), so an individual must display the appropriate behavioral phenotype for their environment. Thus, an aggressive individual will do well when competition is high, but poorly when predator density is high, and maternal effects may be an adaptive means by which females can change the behavioral phenotype of offspring in preparation for the environment (Mousseau and Fox 1998).

However, not all studies show a positive relationship between maternal competition and yolk testosterone (e.g., Groothuis and Schwabl 2002; Verboven et al. 2005; Gil et al. 2006; Safran et al. 2008; Schmaltz et al. 2008) and these studies are commonly regarded as anomalies. Furthermore, we do not know what mechanisms mediate the effects of prenatal testosterone on offspring behavior. Thus, my dissertation research has two primary objectives: 1) determine the causes of interspecies variation in yolk testosterone allocation in competitive environments and 2) explore the molecular mechanisms facilitating behavioral plasticity in offspring exposed to prenatal testosterone. Specifically, I performed a meta-analysis to identify species-specific traits influencing yolk testosterone allocation in response to competition (Chapter 2) and then experimentally tested the findings (Chapter 3). Next, I explored natural variation in molecular

responses to yolk testosterone in a wild songbird species (Chapter 4) and then experimentally tested the mechanisms in a captive songbird species (Chapter 5).

Interspecific variation in yolk testosterone response

Despite several studies failing to find a positive relationship between maternal competitive environment and yolk testosterone allocation (Groothuis and Schwabl 2002; Schmaltz et al. 2008; Verboven et al. 2005; Cariello et al. 2006; Safran et al. 2010; Remeš 2011; Welty et al. 2012; Paquet et al. 2013; van Dijk et al. 2013; Santos 2016), few have attempted to address why this variation exists. Those that have suggest these contradictory results are an artefact of their measure of competition being confounded by other factors (Groothuis and Schwabl 2002) or that their findings were skewed by life-history traits, such as a high incidence of extra-pair mating (Welty et al. 2012) or colonial living (Paquet et al. 2013). Interspecific variation in yolk testosterone could exist due to evolutionary constraints arising from life-history traits. Innate differences in competitive environments due to life-history traits could shape how a species responds to transient changes in competition, such as seasonal variation in breeding density. However, few studies have explored interspecific variation in yolk testosterone. Past comparative approaches have examined how average yolk testosterone concentrations are related to traits like nestling development (Gorman and Williams 2005; Gil et al. 2007; Schwabl et al. 2007), aspects of song (Garamszegi et al. 2007), and coloniality (Gil et al. 2007). However, investigation of the evolution of a purportedly adaptive maternal effect requires consideration of environmental context, such as change in maternal-derived yolk testosterone values rather than average concentrations, and this has yet to be explored.

Sources of variation in aggressive behaviors

In order to understand how maternal hormones influence behaviors, we must first know what factors create behavioral variation. Behavioral studies have been biased toward the hypothalamic-pituitary-gonadal (HPG) axis, specifically testosterone (Soma 2006), since Berthold's (1894) classic study in roosters showed that castration decreases aggressive behaviors. Thus, it is not surprising that the few studies that have attempted to test mechanisms by which yolk testosterone functions focused on testosterone and its androgen receptor. Pfannkuche et al. (2011), for example, found that offspring exposed to higher yolk testosterone had lower plasma testosterone and androgen receptor expression in whole brain tissue. However, testosterone does not restrictively act through androgen receptors, but can also be converted to estrogen via aromatase and bind to the estrogen receptor (Groothuis and Schwabl 2008). Recent evidence suggests estrogen receptors may play a larger role in aggressive behaviors than the androgen receptor (Soma 2006). Aggressive phenotype covaries with neural expression of estrogen receptor alpha (Rosvall et al. 2012), and aromatase inhibitors and estrogen receptor alpha antagonists also lower aggression (Walters and Harding 1988; Schlinger and Callard 1990). Therefore, yolk testosterone could also utilize estrogen receptors to mediate its affect on offspring aggression. In support of this, neither yolk injections of dihydrotestosterone (an unaromatizable metabolite of testosterone that only uses androgen receptors; Hegyi and Schwabl 2010) nor an androgen receptor antagonist (Müller et al. 2005) affected offspring.

Sources of variation in aggressive behaviors beyond the HPG axis

Advances in genomics have given us the tools to expand studies examining the molecular mechanisms that regulate behaviors (Crews 2008; Wong and Hofmann 2010). Variation in

aggression has now been attributed to gene regulation in the brain associated not only with the HPG axis, but also the hypothalamic-pituitary-adrenal (HPA) axis, serotonin, nitric oxide, dopamine, and the hypothalamic-neurohypophysial-system (Nelson and Trainor 2007; Mukai et al. 2009; Filby et al. 2010). Therefore, long-lasting variation in aggressive behavior could be a result of prenatal testosterone acting on multiple neural pathways across the brain. Recent experimental evidence suggests that the maternal environment (predation risk) can influence genome-wide expression of genes in embryos (Mommer and Bell 2014). Moreover, genes in these behaviorally sensitive pathways are present during prenatal development and are sensitive to steroids (Smeets and González 2000; Bethea et al. 2002; Perlamn and Arnold 2003; Perlman et al. 2003; Ahmed et al. 2014), making them candidates for manipulation by yolk testosterone to alter offspring aggression long-term.

Potential role of epigenetic mechanisms

Yolk testosterone causes long-term variation in aggressive phenotypes (e.g., Strasser and Schwabl 2004; Müller et al. 2009), and if behavioral phenotypes are regulated by the expression of genes (Filby et al. 2010; Rosvall et al. 2012), then epigenetic modifications offer a promising explanation for how maternal environments can generate long-lasting variation in gene expression. Epigenetic effects change gene expression without altering DNA sequence; e.g., adding methyl groups to cytosines at CG dinucleotides to suppresses gene expression (Holliday 1994). Methlylation marks are programmed during early development (Vickaryous and Whitelaw 2005), which coincides with embryonic exposure to maternal testosterone (von Engelhardt et al. 2009). Therefore, DNA methylation may be the mechanism mediating the effects of yolk testosterone on behaviorally sensitive genes and, thus, behaviors. Indeed,

maternal effects occurring in the early postnatal environment have been shown to affect offspring DNA methylation (Weaver et al. 2004; Champagne et al. 2006; Murgatroyd et al. 2009). Yet, few studies have examined if the prenatal environment can exert similar epigenetic effects (i.e., methylation of offspring HPA axis and pre-breeding rainfall, Rubenstein et al. 2015; methylation of adult growth factors and maternal nutrition, Heijmans et al. 2008).

Summary of dissertation chapters

In Chapter 2, I performed a meta-analysis to identify species-specific traits influencing yolk testosterone allocation. I used yolk testosterone allocation effect size in response to maternal competition in 25 intraspecific avian studies and tested whether and how different life-history traits (e.g., coloniality, nest type, mating type, and parental effort) account for variation in yolk testosterone allocation. If intraspecific variation in yolk testosterone allocation has evolved to be adaptive for competitive environments, I expect to find this maternal effect related to life-history traits that potentially influence interspecific variation in competition.

In Chapter 3, I experimentally tested the hypothesis that a colonial species would deviate from the pattern of increasing yolk testosterone in response to conspecific aggression. I collected two clutches of eggs from zebra finch (*Taeniopygia guttata*) pairs laid in untreated control clutches and experimental clutches laid during conspecific intrusions. If interspecific variation in yolk testosterone allocation has evolved to be adaptive for life-history traits influencing competition (e.g., coloniality), I predict that a colonial species will respond to increased aggression without increasing yolk testosterone.

In Chapter 4, I investigated relationships between natural variation in the competitive environment experienced by wild songbirds (Eastern bluebirds, *Sialia sialia*), their yolk

testosterone allocation, and offspring phenotype (i.e., growth and neural estrogen receptor alpha DNA methylation). I hypothesized that (1) higher breeding densities would be positively correlated with testosterone allocation to egg yolks and (2) higher yolk testosterone concentrations would be positively correlated with offspring growth and negatively correlated with estrogen receptor alpha DNA methylation in offspring brain tissue.

In Chapter 5, I further explored the molecular mechanisms mediating maternal effects by examining genome-wide changes in gene expression (using RNA-seq) in two socially sensitive brain regions (nucleus taenia of the amygdala and hypothalamus) in both male and female zebra finches using egg injections of testosterone or the vehicle (control). I tested the hypothesis that experimentally increased yolk testosterone would increase aggressive behaviors in both sexes, and that these behavioral changes would be accompanied by genome-wide changes in expression of behaviorally sensitive genes.

Altogether, this dissertation examines previously unexplained variation in maternal effects and the mechanisms that mediate them. This work includes the first use of a comparative approach to examine a maternal effect using effect sizes (i.e., maternal response to competitive environments) rather than species average values. This work is also one of the first to examine DNA methylation and genome-wide changes in gene expression as a mechanistic mediator between prenatal hormones and long-lasting changes in social behavior. Ultimately, this work helps elucidate the adaptive role of maternal effects and helps us predict how species will respond to changes in their social environment. Furthermore, this work clarifies what molecular mechanisms mediate the effects of maternal testosterone on offspring, helping us better understand the environmental and molecular causes of phenotypic plasticity.

CHAPTER 2

EVOLUTIONARY IMPLICATIONS OF INTERSPECIFIC VARIATION IN A MATERNAL EFFECT: A META-ANALYSIS OF YOLK TESTOSTERONE RESPONSE TO COMPETITION[A]

[A] Bentz, A.B., Becker, D.J. and Navara, K.J. 2016. Evolutionary implications of interspecific variation in a maternal effect: a meta-analysis of yolk testosterone response to competition. R. Soc. Open Sci. 3:160499. Reprinted here with permission of publisher.

ABSTRACT

Competition between conspecifics during the breeding season can result in behavioural and physiological programming of offspring via maternal effects. For birds, in which maternal effects are best-studied, it has been claimed that exposure to increased competition causes greater deposition of testosterone into egg yolks, which creates faster growing, more aggressive offspring; such traits are thought to be beneficial for high-competition environments. Nevertheless, not all species show a positive relationship between competitive interactions and yolk testosterone, and an explanation for this interspecific variation is lacking. We here test if the magnitude and direction of maternal testosterone allocated to eggs in response to competition can be explained by life-history traits while accounting for phylogenetic relationships. We performed a meta-analysis relating effect size of yolk testosterone response to competition with species coloniality, nest type, parental effort, and mating type. We found that effect size was moderated by coloniality and nest type; colonial species and those with open nests allocate less testosterone to eggs when in more competitive environments. Applying a life-history perspective helps contextualize studies showing little or negative responses of yolk testosterone to competition and improves our understanding of how variation in this maternal effect may have evolved.

Key words: avian, life-history traits, coloniality, nest type, aggression

INTRODUCTION

Competition is a selective force continuously shaping individual phenotypes and populations (West-Eberhard 1983; Schluter 2000; Wilson 2014). Aggression—or the threat of aggression—is usually needed to be successful in competitive interactions (West-Eberhard 1983), and while aggression can be beneficial if it secures resources, there is invariably a cost (e.g., time and energy or increased risk of injury and pathogen transmission, Brown 1964; Hawley et al. 2011). The strength of competitive aggression as a selection pressure can depend on variation in the social environment (e.g., breeding density, Male et al. 2006), and this environmental heterogeneity can potentially elicit adaptations that span generations via maternal hormones (von Engelhardt and Groothuis 2011). Hormone-mediated maternal effects are a non-genetic source of phenotypic variation in offspring exposed to maternal hormones during periods of high developmental plasticity, such as embryonic development (Mousseau and Fox 1998; Wolf and Wade 2009). Maternal effects are common across taxa (e.g., mammals, Dloniak et al. 2006; and fish, Burton et al. 2011), but are best studied in birds because maternal hormones are deposited in externally developing eggs (von Engelhardt and Groothuis 2011). The majority of studies examining intraspecific variation in avian maternal hormone allocation focus on the effects of competitive social interactions (von Engelhardt and Groothuis 2011). Most of these studies find a positive relationship between testosterone allocated to egg yolks and transient increases in competition, such as increased breeding density (Schwabl 1997; Reed and Vleck 2001; Pilz and Smith 2004; Eising et al. 2008; Bentz et al. 2013; Duckworth et al. 2015) or conspecific aggression (Whittingham and Schwabl 2002; Mazuc et al. 2003; Navara et al. 2006b; Hargitai et al. 2009; Guibert et al. 2010). Exposure to increased yolk testosterone typically increases nestling development (e.g., Schwabl 1996a; Eising et al. 2001; Navara et al. 2005;

Navara et al. 2006a; Müller et al. 2008; but see von Engelhardt and Groothuis 2011) and aggressive behaviours throughout life (Strasser and Schwabl 2004; Eising et al. 2006; Parteck and Schwabl 2008). This has led researchers to postulate that yolk testosterone allocation is an adaptation to competitive environments (Groothuis et al. 2005).

Maternal effects may be an adaptive means by which females change the phenotype of offspring in preparation for the current environment (Groothuis et al. 2005; von Engelhardt and Groothuis 2011), specifically by increasing juvenile survival in the maternal environment (Pilz et al. 2004) and/or success as adults if they remain in or select a similar habitat to that of the maternal environment (Brown and Brown 2000). The phenotypic changes in offspring associated with yolk testosterone, like increased aggression, seem beneficial in high-competition environments; however, not all studies show a positive relationship between competitive environment and yolk testosterone (Groothuis and Schwabl 2002; Schmaltz et al. 2008; Verboven et al. 2005; Cariello et al. 2006; Safran et al. 2010; Remeš 2011; Welty et al. 2012; Paquet et al. 2013; van Dijk et al. 2013; Santos 2016). Authors of one study showing a negative relationship between breeding density and yolk testosterone suggested these results were an artefact of more aggressive birds defending larger territories and creating lower densities; alternatively, their measure of density could have been confounded with vegetation height, making it difficult to interpret the results (Groothuis and Schwabl 2002). Yet, other studies have also failed to support a positive relationship between yolk testosterone and competition, and authors have offered varying reasons for these findings, including high incidence of extra-pair mating (Welty et al. 2012) and coloniality (Paquet et al. 2013). Life-history traits could plausibly cause innate differences in competitive environments that shape how a species responds to transient changes in competition, such as seasonal variation in breeding density. Furthermore, to

fully determine if this maternal effect is an adaptation to transient changes in competition, a comparative phylogenetic approach is necessary to show how maternal effects have evolved with changes in innate levels of competition across species (Harvey and Pagel 1991).

Interspecific variation in yolk testosterone may exist due to evolutionary constraints arising from life-history traits; however, few studies have explored interspecific variation in yolk testosterone. Those that have taken comparative approaches showed that average yolk testosterone concentrations per bird species are related to nestling development (Gorman and Williams 2005; Schwabl et al. 2007) and aspects of songs (Garamszegi et al. 2007). Gil et al. (2007) performed one of the most comprehensive comparative studies and showed that colonial bird species allocate more yolk androstenedione, the biologically inactive precursor to testosterone, but not testosterone, than solitary species. Though this body of work has examined species differences in average yolk testosterone concentrations, investigation of the evolution of a purportedly adaptive maternal effect requires consideration of environmental context; therefore, change in maternal-derived yolk testosterone values rather than average concentrations are required. We present here the first comparative approach of a maternal effect by asking if effect size (i.e., maternal response to competitive environments) varies across species based on life-history traits that evolved with potentially different innate levels of competition. To test this, we performed a meta-analysis with yolk testosterone allocation effect size in response to competition in 25 intraspecific avian studies. We tested whether and how different life-history traits account for interspecific variation in yolk testosterone allocation. If intraspecific variation in yolk testosterone allocation has evolved to be adaptive for competitive environments, we would expect to find this maternal effect related to life-history traits that potentially influence interspecific variation in competition.

METHODS

Literature search

We performed systematic searches in Web of Science, Google Scholar, JSTOR, and PubMed. We used the same string of search terms in all databases: (avian OR bird) AND ("yolk testosterone" OR "egg testosterone" OR "yolk androgen" OR "egg androgen") AND (aggression OR "simulated territorial intrusion" OR "breeding density" OR "social environment" OR "colony size" OR "maternal environment"). We restricted searches from the date of the search, September 5, 2016, back to 1993, when the first discovery of maternal yolk testosterone allocation was made by Schwabl et al. (1993). We only included observational or experimental studies comparing yolk testosterone allocation across variation in environments eliciting competitive behaviours in breeding females following a systematic exclusion process (Moher et al. 2009; see Appendix, Figure S2.1). Studies that only presented data for androstenedione, the biologically inactive precursor to testosterone (e.g., Gil et al. 2006), or examined yolk testosterone relationships with social hierarchy, which is not an environmental manipulation (e.g., Müller et al. 2002; Tanyez et al. 2008), were excluded.

We ultimately included 22 articles in our meta-analysis. Two articles (Groothuis and Schwabl 2002; Bentz et al. 2013) performed studies in two different years and analyzed these data separately, so we included both years. We also augmented our sample size with an unpublished dataset (A.B. Bentz, V.A. Andreasen, and K.J. Navara, unpublished data) involving the experimental manipulation of zebra finch (*Taeniopygia guttata*) pairs' competitive environment using conspecific intrusions (n = 16 pairs) to bring the final sample size to 25 records. Our criteria for inclusion in the meta-analysis resulted in a small sample size, and conclusions regarding yolk testosterone allocation response to competition should be regarded

cautiously. However, given the low number of available studies, we hope our meta-analysis will prompt future avian work within a new life-history and phylogenetic framework.

Effect size calculation

We reported effect sizes as the correlation-based *r* between our factor (i.e., competitive aggression) and response (i.e., yolk testosterone allocation). In most cases *r* was not reported; therefore, we followed Rosethal and DiMatteo (2001) to convert test statistics (e.g., *F*, *t*, or χ^2) into *r*. We primarily calculated *r* using *F* and the error df, when the numerator df was 1:

$$r = \sqrt{\frac{F}{F + df_{error}}}$$

If there was more than one df in the numerator or a random effect was included, we converted the reported *p*-value to a standard normal deviate *Z*-score and used the sample size to obtain *r*:

$$r = \frac{Z}{\sqrt{N}}$$

For our analyses, we assigned a negative value to effect sizes for which the independent variable was negatively related to yolk testosterone. However, we were unable to determine directionality of effect for four studies with non-significant results (e.g., only a *F* statistic and *p*-value were reported); therefore, we performed our primary analyses with both negative and positive values, though only results from the case of positive values are depicted. Directional *r* effect sizes were converted to Fisher's *Z* to stabilize variance using R package *metafor* (Viechtbauer et al. 2010).

Selection and coding of moderator variables

We selected moderators based on previous interspecific comparisons (Gorman and Williams 2005; Garamszegi et al. 2007; Gil et al. 2007; Schwabl et al. 2007) and life-history

traits that have the potential to influence the adaptive context for the direction or strength of the relationship between level of competition and yolk testosterone response (i.e., effect size). Coloniality (i.e., solitary, semi-colonial, or colonial) can influence the frequency of intraspecific competition (Alexander 1974; Sachs et al. 2007), nest type (i.e., open- vs. closed-nesters) can influence nest competition and predation risk (Martin and Li 1992; Newton 1994; Fisher and Wiebe 2006; Fontaine et al. 2007), parental investment (i.e., a multiple correspondence analysis of clutch size, altricial vs precocial nestlings, and time to fledge; DeLeeuw et al. 2009) needed in biparental species (none of the species in this study exhibit male- or female-only care) can influence intra-sexual mate competition (Sandell 1998; Cain 2014), and mating type (i.e., polygamous/cooperative breeders vs. monogamous) can affect intra-sexual mate competition (Petrie and Kempenaers 1998; see Appendix A for references and details on moderator coding, Table S2.1). Because monogamous species are not always truly monogamous (Griffith et al. 2002), we also calculated degree of monogamy when data were available (n = 10 species) using the weighted average of percentage of nests that have extra-pair young across a minimum of two populations (see Appendix A, Table S2.1). We also considered experiment type, as studies differed in how they measured and/or elicited competitive aggression (correlative, no manipulation; indirect manipulation of aggressive interactions by changing environment; or direct manipulation of aggressive interactions with simulated territorial intrusions; STI).

Model selection and hypothesis testing

We used random effect models (REM) to estimate the average true effect size and heterogeneity among effect sizes, and univariate mixed-effects models (MEM) to test how each life-history trait moderates the relationship between competition and yolk testosterone response

(Gurevitch and Hedges 1999). Separate MEMs with observation, study, and species included as random effects (see "Controlling for phylogenetic signal" below for details on the species component) were built to account for unit-level heterogeneity, study pseudoreplication, and phylogeny while explaining variation in effect size according to moderator variables (Konstantopoulos 2011; Nakagawa and Santos 2012), using the *metafor* package in R (Viechtbauer et al. 2010). To assess relative support for each hypothesis, we first fit models using maximum likelihood and calculated AICc to correct for the small sample size (Burnham and Anderson 2003). We next refit these models using restricted maximum likelihood to obtain unbiased estimates of variance components and tested if each moderator explained significant heterogeneity with Cochran's *Q*. We also assessed the relative contribution of true heterogeneity to the total variance in effect size through the I^2 statistic (Higgins and Thompson 2002; Nakagawa and Santos 2012). To quantify the variation in effect size explained per moderator, we calculated the proportional reduction in the summed variance components from each MEM compared to the summed variance components of the REM, equivalent to a pseudo-R^2 value (Raudenbush 2009; López-López et al. 2014). For models within 2 ΔAICc of the best-fit model (Burnham and Anderson 2003), we performed post-hoc comparisons adjusting for the potentially inflated false-discovery rate using the Benjamini and Hochberg correction (Benjamini and Hochberg 1995; Hothorn et al. 2008). In a secondary analysis, we built a MEM for percent extra-pair copulations and performed the same set of analyses, as data for this moderator was available for a subset of records (n = 16 records; 10 spp.). We tested residuals for normality and examined profile likelihood plots of variance components to ensure parameter estimates were identifiable.

Meta-analysis models include weighting by sampling variances, which results in more precise estimates of coefficients and increases power, even when sample size is small (Cohn and

Becker 2003; Valentine et al. 2010). However, due to our small sample size, we limited the number of models to under one-third of our *n* (Burnham and Anderson 2003).

Controlling for phylogenetic signal

Closely related species may have similar yolk testosterone responses (Freckleton et al. 2002). We first obtained a phylogeny using R package *ape* to prune the complete tree of life to our 17 species (Paradis et al. 2004; Hinchliff et al. 2015). The mean effect size per species was calculated by weighting each observation by the corresponding sample size. We determined phylogenetic signal using maximum likelihood to estimate Pagel's λ (Pagel 1999) and compared the fit of this model against those in which λ was 0 (phylogenetically uncorrelated) and 1 (phylogenetically dependent) using likelihood ratio tests in R package *geiger* (Freckleton et al. 2002; Harmon et al. 2008). We found evidence for phylogenetic dependence in effect size (Figure 2.1). The maximum likelihood estimate of λ was 1, and likelihood ratio tests suggested this estimate did not differ from the Brownian motion model of trait evolution ($\chi 2 < 0.001$, df = 1, $p = 1.00$). Yet, we could also not reject that λ departed from a model with no phylogenetic signal ($\chi 2 = 1.21$, df = 1, $p = 0.27$). To include all 25 records while accounting for phylogenetic non-independence in the above MEM analyses, the covariance structure of the species random effect was specified by the correlation matrix of our phylogeny, equivalent to a phylogenetic meta-analysis (Viechtbauer et al. 2010; Konstantopoulos 2011; Nakagawa and Santos 2012).

Publication bias

We also tested for publication bias, the preferential publication of significant over non-significant results or those with a small effect size (Rosenthal 1979). We used a funnel plot to

visualize potential bias, where a symmetrical funnel suggests little bias (Egger et al. 1997). We assessed symmetry with regressions to test associations between effect sizes and sampling variances (Sterne and Egger 2005). We then used the trim-and-fill method to estimate the number of observations missing owing to publication bias and tested if addition of these points influenced REM estimates (Duval and Tweedie 2000; Viechtbauer et al. 2010).

RESULTS

Effect of moderators

We found significant heterogeneity in avian yolk testosterone responses to competitive environments (I^2 = 0.92, Q = 95, df = 24, $p < 0.0001$; Figure 2.2), with 52% of studies finding that females significantly increase yolk testosterone in more competitive environments (n = 13), 12% of studies finding significant decreases (n = 3), and 36% finding no significant effect (n = 9). The REM showed an average positive but non-significant effect size ($\mu = 0.28$, $z = 0.83$, $p = 0.41$), likely owing to the large variance attributed to phylogeny ($\sigma^2_1 = 0.44$).

Model comparison by AICc lent the most support for species coloniality and nest type as the strongest predictors of yolk testosterone response to competition, as the cumulative Akaike weight of these models exceeded 90% (Table 2.1a). Coloniality explained 55% of the variation in effect size compared to the base REM (Q_M = 14.85, df = 2, $p < 0.001$), and nest type explained 29% of effect size variation (Q_M = 6.70, df = 1, p = 0.01; Table 2.1a). Only these two moderators performed better than the REM when compared to other species- and study-specific moderators. The ranking of models by AICc was identical when we assigned negative signs to the four effect sizes where directionality could not be determined and lent even more support to coloniality as the best predictor of effect size. Thus, the presented results using positive values for those four

effect sizes could be interpreted as conservative. Our secondary analysis for degree of monogamy (% EPC) found that this trait was not associated with effect size (Table 2.1b). All model residuals were normally distributed ($W \geq 0.92$, $p \geq 0.06$) with the exception of degree of monogamy ($W = 0.81$, $p < 0.01$), and profile likelihood plots of the variance components indicated strong parameter identifiability. Variance for the species-level random effect (σ^2_1) ranged from 0.20 to 0.49. In contrast, variance components for the study- and observation-level random effects (σ^2_2 and σ^2_3, respectively) were consistently zero. Accordingly, a large proportion of the unaccounted variance in effect size was due to residual heterogeneity from phylogeny ($I^2_{species}$ ranged from 0.82 to 0.92).

Our best-supported MEMs independently included coloniality and nest type. Adjusting for multiple comparisons, we found that colonial species had lower yolk testosterone responses than both solitary and semi-colonial species (all $z < -2.6$, $p < 0.02$; Figure 2.3a). We likewise found a strong overall effect of nest type on yolk testosterone response to competition, with lower effect sizes for species with open nests than closed nests ($z = -2.59$, $p = 0.01$; Figure 2.3b).

Publication bias

We found little evidence of publication bias in the study of avian yolk testosterone response to competition (see Appendix A, Figure S2.2). We did not detect an association between effect size and standard error ($z = 0.80$, $p = 0.42$), and trim-and-fill analyses using the R0 estimator did not detect any missing effect sizes. These results suggest a lack of the "file drawer" effect (Rosenthal 1979); research in this field was earnestly published regardless of effect size or statistical significance.

DISCUSSION

Interspecific variation in female testosterone allocation to eggs as a response to competitive environments was strongly predicted by two life-history traits, coloniality and nest type. It should be noted that no colonial breeders also had closed nest types in our dataset; therefore, despite analyzing these separately, it may be difficult to say which of the two life-history traits is truly the stronger driver of effect size. However, the R^2 for coloniality was far higher than that of nest type, supporting the argument that coloniality is the stronger of the two. The majority of colonial species (3 of 5 species) in our dataset are altricial, meaning only two colonial species were also precocial. Given the current sample size, we cannot test for an interaction between coloniality and development type; however, the adaptive value of yolk testosterone could vary as precocial nestlings face direct competition from peers in higher densities (unlike altricial nestlings). Additional data for precocial species are therefore needed. Nevertheless, we more broadly found no support for development type as a determinant of effect size, suggesting the relationship between coloniality and yolk testosterone allocation in response to competition is driven more directly by features of colonial life history. That experiment type was not a strong predictor of this maternal effect also suggests correlative studies were able to obtain an adequate range of competition and that those studies directly inducing competition did so within the natural range. Further, strong phylogenetic signal in effect size provides support for selection on yolk hormone allocation strategy as an adaptation to species-specific competition.

Coloniality

The life-history trait that received the most support in our analysis was coloniality. Colonial species deposited less yolk testosterone in response to competition than either solitary

or semi-colonial species. Solitary and semi-colonial species did not differ in yolk testosterone response, though there was a trend for semi-colonial species to deposit less yolk testosterone. While we did not include Gil et al. (2006) in the meta-analysis because they reported the relationship between colony size and yolk androstenedione, not testosterone, it is interesting to note that they did not find a significant relationship between yolk androstenedione and colony size in the highly colonial barn swallow (*Hirundo rustica*).

The costs and benefits associated with colonial breeding create trade-offs and selective pressures unique from those under which solitary species have evolved. Most notably, colonial species have a higher frequency of conspecific interactions (Birkhead 1978; Klatt et al. 2004; Sachs et al. 2007). Aggression between conspecifics can lead to physical harm and increase reproductive costs, such as egg loss (Caraco 1979; Burger and Gochfeld 1990). Thus in colonial species, for which opportunities for competitive interactions are much more frequent, the potential adaptive value of reducing the severity of these aggressive interactions should be high. For example, current fitness is higher in colonial birds that allopreen neighbours (an altruistic behaviour) at a higher rate and have fewer fights than those that do not express this behaviour (Lewis et al. 2007). Indeed, there are several examples of adaptations in social species that decrease severity of aggression compared to solitary species, such as increased attack latencies (Pruitt et al. 2012), increased number of appeasement signals (Birkhead 1978), and altered neural nonapeptide responses to aggression (Goodson and Kingsbury 2011). The transition to coloniality could have created selection for a decrease in amplitude of aggressive actions, which would be facilitated by a lower yolk testosterone response creating offspring with less aggressive phenotypes. Within many colonial species, increases in breeding density are accompanied by more frequent interactions with neighbours but fewer interactions escalate to high levels of

aggression (Birkhead 1978; Druzyaka et al. 2015). In contrast, aggression is less frequent for solitary species, and the burden of the cost is dispersed over time so the adaptive value of being aggressive is likely much higher (Rosvall 2008). This would favour increased yolk testosterone in response to competitive environments to increase offspring aggressive phenotype.

Another explanation for attenuated yolk testosterone responses in colonial species could be selective pressures imposed by greater parasite risk. Colonial species live in close proximity to one another and have a higher risk of pathogen infection and ectoparasitism, which has direct fitness effects on mortality and more subtle effects on fecundity (Tella 2002; Brown and Brown 2004a). There could be strong selection to allocate less yolk testosterone when colony size is large and risk of parasitism is high, because yolk testosterone may decrease immunity and increase susceptibility (Navara et al. 2005; Rutkowska et al. 2007; von Engelhardt and Groothuis 2011).

Nest type

Species with open nest types deposited less yolk testosterone in response to competition than those nesting in closed nests, like cavities. One explanation for this could be that birds with open nests suffer greater nest predation than those nesting in cavities (Martin and Li 1992; Fontaine et al. 2007). Predation tends to select against bold, aggressive individuals and forces individuals to re-allocate time and energy away from competition toward predator avoidance (Fisher and Wiebe 2006; Bell and Sih 2007). Females of species exposed to greater levels of nest predation may have adapted a more subtle response to competitive challenges, as shown here. An experimental study found females exposed to frequent predation during egg laying deposit less testosterone into their eggs (Coslovsky et al. 2012). Yolk testosterone generally increases growth

and begging rates, so by not allocating more yolk testosterone despite having a more competitive environment, females could be using an adaptive strategy to reduce revealing traits (i.e., larger, louder offspring) and thus avoid detection by predators (McDonald et al. 2009). However, a meta-analysis of Passeriformes suggests that yolk testosterone allocation may increase with predation rate, possibly to accelerate development and reduce exposure to predation in the nest (Schwabl et al. 2007). Thus, an alternative explanation for our findings is that cavity-nesting birds may compete more for limited nest sites compared to open-nesting birds (Newton 1994) and therefore have adapted strategies to increase aggressive phenotypes when nest site competition is high (Rosvall 2008), such as increasing yolk testosterone with breeding density.

Potential mechanisms

Researchers have previously postulated that female plasma and yolk hormone responses to aggression were positively correlated (Schwabl 1996b; Jawor et al. 2007), leading to the assumption that competition should increase yolk testosterone. Yet not all studies show a positive relationship between plasma and yolk testosterone in response to aggression (Navara et al. 2006b; Verboven et al. 2003). Furthermore, there is a trend for both solitary and colonial species to increase plasma testosterone with conspecific aggression (Birkhead 1978; Smith et al. 2005), yet we show here that these groups differ in yolk testosterone allocation. Colonial females also tend to have higher circulating testosterone than solitary females (Møller et al. 2005), but their egg yolks do not have higher average concentrations of testosterone (Gil et al. 2007). The mechanisms regulating yolk and plasma testosterone could therefore be independent (Hackl et al. 2003; Okuliarova et al. 2011). For example, differences in expression of follicular steroidogenic enzymes, such as aromatase, explain substantial variation in yolk testosterone but not in plasma

testosterone (Egbert et al. 2013). Expression of steroidogenic enzymes can change rapidly in response to environmental factors (Payne and Hales 2004; Egbert et al. 2013) and may be a point of selection for yolk testosterone response to competition. In contrast, plasma testosterone may originate from multiple sources, such as the gonad and adrenal glands, and new research asserts that steroids can be produced rapidly and locally in the brain in response to aggression (Soma et al. 2008; Schlinger and Remage-Healey 2012), which would operate independent of follicular production. Other factors could also influence yolk allocation independent of plasma, such as metabolic conjugation (Paitz and Bowden 2008) or enzymatic barriers or membrane transporters to alter steroid transfer from follicles to the yolk (Moore and Johnston 2008). There is also evidence that natural selection can shape yolk and plasma hormone allocation separately. Yolk testosterone concentrations have a heritable component (e.g., Gil and Faure 2007; Groothuis et al. 2008; Tschirren et al. 2009a), and yolk and plasma testosterone respond to artificial selection differently (Okuliarova et al. 2011).

If yolk and plasma testosterone are moderated independently and have a heritable component, then selection on mechanisms moderating plasma testosterone may have occurred to benefit females as they respond to aggressive interactions; while, selection on mechanisms moderating yolk testosterone may have occurred more in response to offspring success. Few studies have explicitly tested fitness consequences (survival or reproductive success) of prenatal exposure to yolk testosterone. There is mixed support for yolk testosterone's effect on early survival in both altricial and precocial offspring (reviewed in von Engelhardt and Groothuis 2011); however, few of these studies incorporated an environmental context or selection pressure known to influence yolk testosterone allocation. One such study found that yolk testosterone increases survival of offspring in poor conditions (Pilz et al. 2004; but see Muriel et al. 2015).

Further, because the effects of yolk testosterone on aggression last into adulthood (Strasser and Schwabl 2004; Eising et al. 2006; Partecke and Schwabl 2008), selection can occur on adult offspring if they remain in or select a similar habitat to that of the maternal competitive environment. There is some evidence habitat choice has a heritable component (Brown and Brown 2000). Regardless, many studies find controversial effects of yolk testosterone on adult survival (Schwabl et al. 2012; Matson et al. 2016) and reproductive success (Vergauwen et al. 2014; Hsu et al. 2016), yet, these studies do not manipulate the environmental context. To further elucidate the life stage in which selection on yolk testosterone allocation may occur, more studies should combine egg hormone injections with environmental manipulations. Studies in other taxa, such as invertebrates and fish, which include strong selection pressures, are able to clearly show the adaptive value of maternal effects (Storm and Lima 2010) and behaviours, like aggression (Bell and Sih 2007).

Future studies

Our analyses illustrate a need to diversify species studied to better understand the role life-history traits play in providing context for the potentially adaptive role maternal effects play. Although we found no evidence of publication bias, this effect has only been studied in 17 species across two decades. Primarily, we identified a lack of hormone-mediated maternal effect research with precocial and non-passerine species. There were also several life-history traits we could not analyze due to sample size limitations, such as trophic level or breeding seasonality, as there are too few studies performed with herbivorous or tropical, year-round territorial species. It would also be interesting to look for indications of divergent evolution in populations of species

that have different life-history strategies; for example, red-necked grebes (*Podiceps grisegena*) can nest in both territorial and colonial patterns (Klatt et al. 2004).

Conclusions

This meta-analysis supports the hypothesis that competition-induced maternal hormone allocation to egg yolks is influenced by life-history traits, suggesting this maternal effect evolved as an adaptation to competition. Colonial species and those with open nests allocate less yolk testosterone in response to competition than solitary and semi-colonial species or species nesting in cavities. Though some of our analyses should be interpreted cautiously owing to sample size limitations, diversifying the number of species studied in future work will only help to further elucidate patterns identified here. Nevertheless, synthetic studies such as ours can help clarify the adaptive role maternally derived hormones play and how they mediate life-history trade-offs. Approaching maternal effects from a life-history perspective will help us understand how variation in this response evolved and elucidate the underlying mechanisms of hormone-mediated maternal effects, an ongoing area of study (Groothuis and Schwabl 2008).

TABLES AND FIGURES

Table 2.1. Univariate rankings of mixed-effects models (MEMs) predicting effect size for the relationship between competitive environment and yolk testosterone response for the (*a*) full and (*b*) reduced dataset. Competing models are ranked by AICc along with the number of model coefficients (*k*); variance components for the species (σ^2_1), study (σ^2_2), and observation random effect (σ^2_3); I^2 statistic, tests of moderator significance (Cochran's *Q* and *p* value); Akaike weights (w_i); and the pseudo-R^2 statistic for each MEM.

Mixed-effects models	***k***	σ^2_1	σ^2_2	σ^2_3	I^2	Q_M	**df**	***p***	**ΔAICc**	w_i	R^2
(*a*) Full dataset (*n* = 25)											
Effect size ~ coloniality	3	0.20	0.00	0.00	0.82	14.85	2	<0.001	0.00	0.78	0.55
Effect size ~ nest type	2	0.32	0.00	0.00	0.88	6.70	1	0.01	2.98	0.17	0.29
Effect size ~ intercept	1	0.45	0.00	0.00	0.92	0.69	1	0.41	6.41	0.03	0.00
Effect size ~ mating type	2	0.48	0.00	0.00	0.92	0.14	1	0.71	9.41	0.01	0.00
Effect size ~ parental effort	2	0.49	0.00	0.00	0.92	0.00	1	0.96	9.57	0.01	0.00
Effect size ~ experiment type	3	0.35	0.00	0.00	0.89	2.49	2	0.29	10.53	<0.01	0.22
(*b*) Reduced dataset (*n* = 16) for percent extra-pair copulations (% EPC)											
Effect size ~ logit(% EPC)	2	0.48	0.00	0.00	0.92	0.53	1	0.47	-	-	0.00

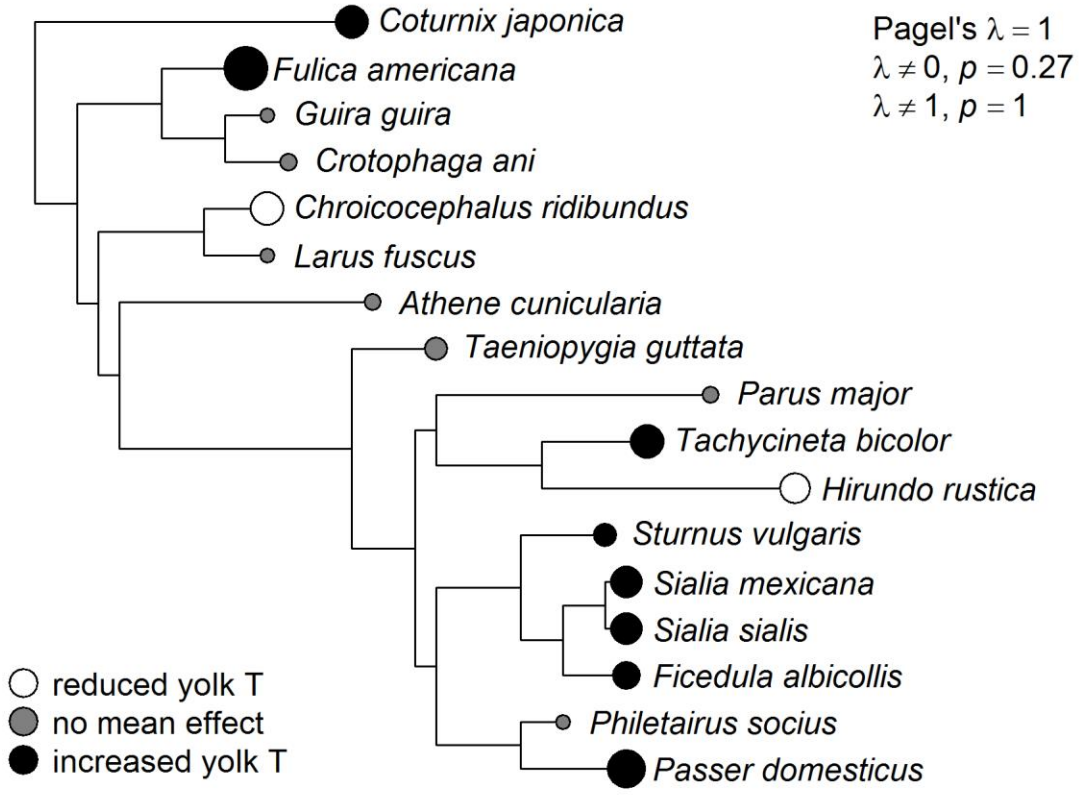

Figure 2.1. Phylogenetic visualization of mean yolk testosterone response per species included in the main analyses. The displayed tree was pruned from the complete tree of life (Hinchliff et al. 2015), with each point representing the weighted average effect size per species. Circle size represents the magnitude of the relationship between yolk testosterone response and competitive environment, and the directionality of this relationship is given in the key and is based on the significance of findings from their respective studies. Results from likelihood ratio tests for phylogenetic signal (Pagel's λ) are provided in the legend.

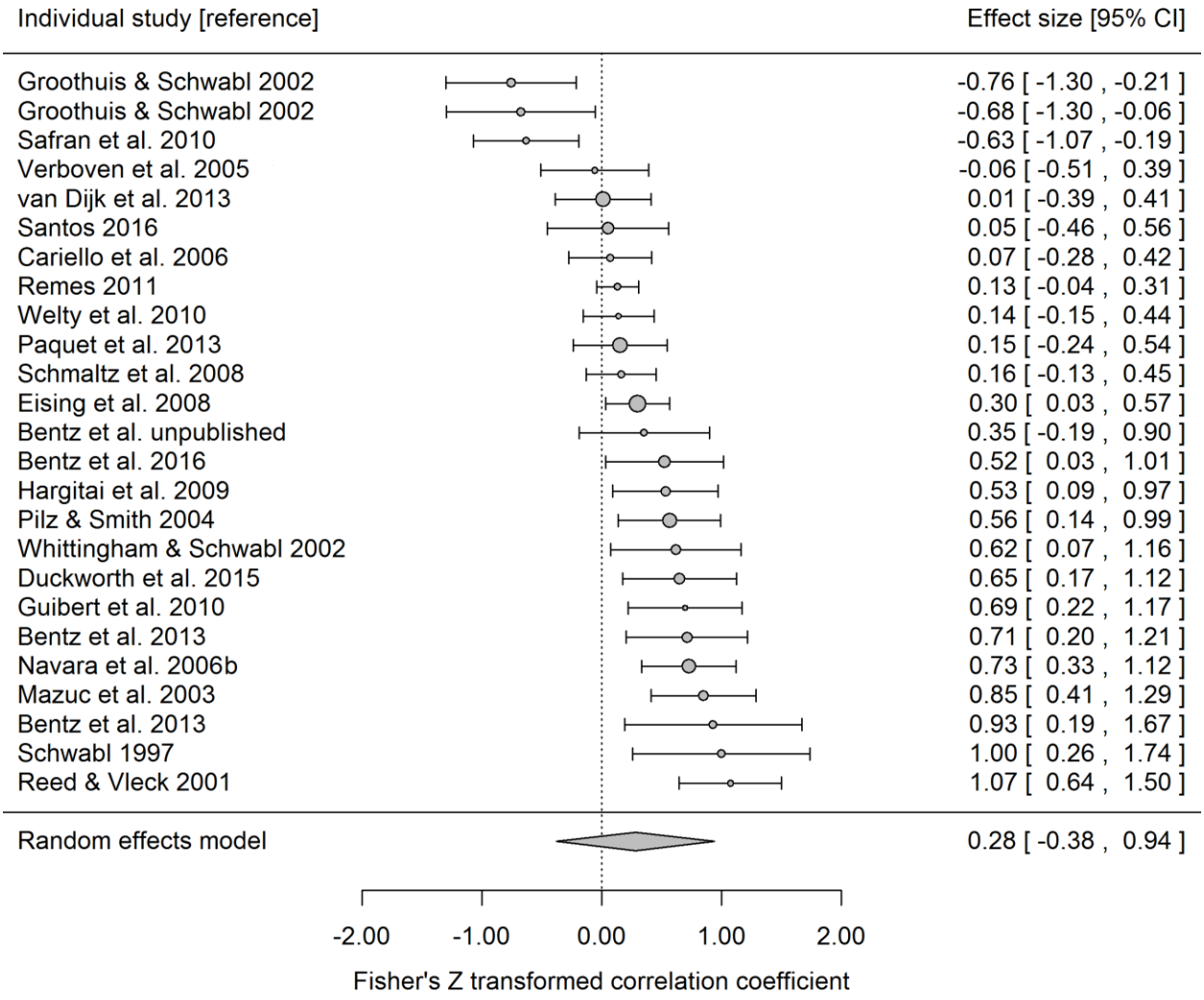

Figure 2.2. Distribution of effect sizes for relationships between competitive environment and yolk testosterone response (Fisher's $Z \pm 95\%$ confidence intervals). Circle size is scaled inversely proportional to the sampling variance, and points to the left of the dashed line indicate cases where competitive environment was associated with decreased yolk testosterone. The diamond displays the estimated true effect from the multilevel REM fit with restricted maximum likelihood.

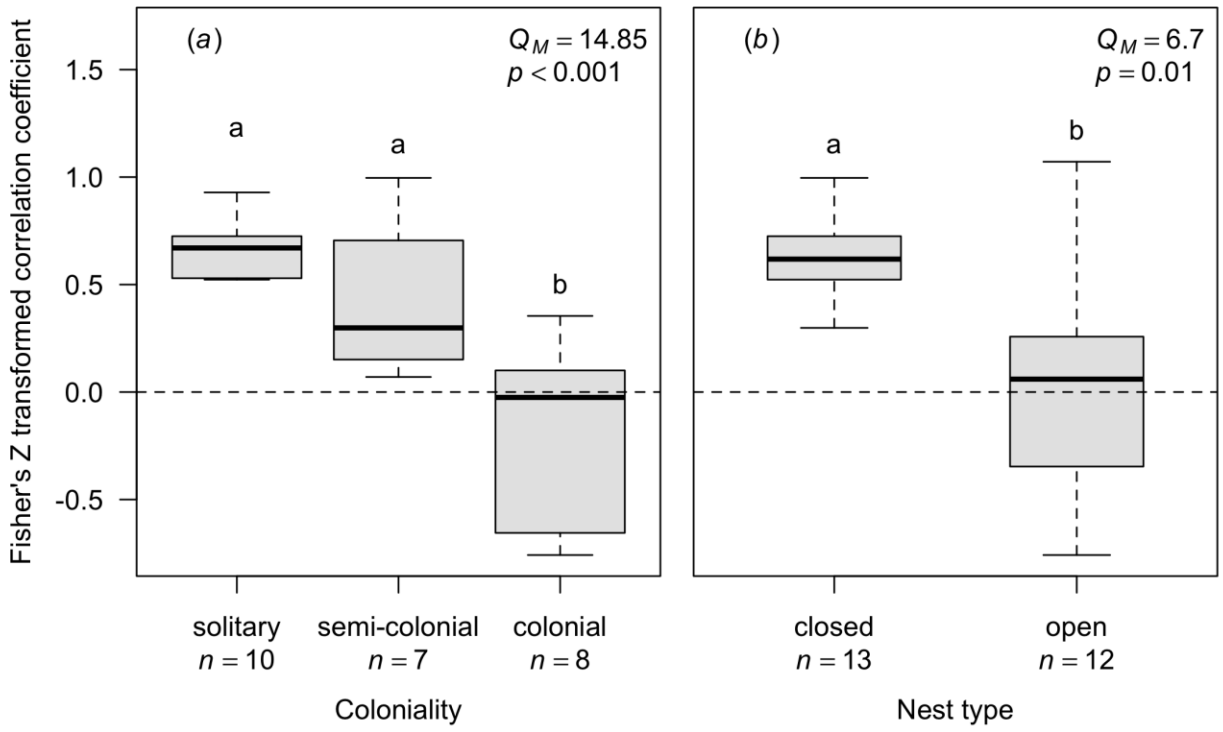

Figure 2.3. Distribution of effect sizes according to (*a*) coloniality and (*b*) nest type. Boxplots show the median and first and third quartile of the effect sizes, and whiskers indicate the range of non-outlier values. The dashed horizontal line represents no yolk testosterone response to competitive environment. Letters indicate significant differences between groups after adjusting for the potentially inflated false-discovery rate using the Benjamini and Hochberg correction. Results of the omnibus tests match those from the full dataset and analyses in table 1. Sample size (*n*) indicates the number of records per category.

CHAPTER 3

THE EFFECTS OF CONSPECIFIC AGGRESSION ON MATERNAL TESTOSTERONE ALLOCATION IN A COLONIAL SPECIES[A]

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ABSTRACT

Maternal hormones can be transferred to offspring during prenatal development in response to the maternal environment, and may adaptively alter offspring phenotype. For example, numerous avian studies show that aggressive competition with conspecifics tends to result in females allocating more testosterone to their egg yolks, and this may cause offspring to have more competitive phenotypes. However, deviations from this pattern of maternal testosterone allocation are found, largely in studies of colonial species, and have yet to be explained. Colonial species may have different life-history constraints causing different yolk testosterone allocation strategies in response to conspecific competition, but few studies have experimentally tested whether colonial species do indeed differ from that of solitary species. To test this, we collected eggs from zebra finches (*Taeniopygia guttata*), a colonial species, in the presence and absence of conspecific intrusions. Females did not alter the concentration of testosterone deposited in eggs laid during intrusions despite becoming more aggressive. These results suggest that maternal effects are not characterized by a uniform response to the social environment, but rather need to be contextualized with life-history traits.

Key words: zebra finch, simulated conspecific intrusion, maternal effect, competition, yolk testosterone

INTRODUCTION

A female's response to her environment can have profound effects not only on her own fitness, but also that of her offspring. Females can allocate hormones to their developing offspring in response to variation in their social environment, and pre-natal exposure to maternal hormones may adaptively alter offspring phenotype (Groothuis et al. 2005). The best-studied effect to date is that of maternal allocation of testosterone in response to competitive aggression in birds. Most research shows that birds increase yolk testosterone in response to aggressive competition with conspecifics, and offspring exposed to elevated levels of yolk testosterone tend to grow more quickly and display more aggressive behaviours (von Engelhardt and Groothuis 2011), which is postulated as being adaptive (Mousseau and Fox 1998; Groothuis et al. 2005).

However, not all studies show a pattern of increasing testosterone allocation with competitive aggression. To help explain this variation, a recent meta-analysis by Bentz et al. (2016a) found that studies of colonial species do not show an increase in yolk testosterone allocation when in more competitive environments (e.g., Verboven et al. 2005), while studies of semi-colonial and solitary species do show increasing concentrations of yolk testosterone (e.g., Schwabl 1997; Bentz et al. 2013). Colonial species have evolved under uniquely different selective pressures from that of solitary species. For instance, colonial species live in close proximity to one another causing them to interact more frequently with conspecifics (Birkhead 1978; Klatt et al. 2004; Sachs et al. 2007) and to have a higher risk of pathogen infection (Brown and Brown 2004a,b). Thus, there may be strong selection for colonial species to avoid increasing concentrations of yolk testosterone in response to competition (e.g., greater densities or interactions) for two potential reasons: (1) this may avoid elevating aggressive phenotypes of offspring that would make them overly aggressive in the frequent interactions they will

ultimately encounter in a colonial setting, and (2) this may avoid the potential immunosuppressive effects of yolk testosterone in a setting where individuals are closely affiliated and could pass pathogens to one another (von Engelhardt and Groothuis 2011). Thus, it is possible that a species' hormonal response to competitive environments is shaped by life-history traits. Unfortunately, studies examining yolk testosterone response to competition in colonial species has largely been correlative; in only one study conducted in a colonial species (i.e., lesser black-backed gull, *Larus fuscus*; Verboven et al. 2005) was competition experimentally altered. Consequently, it is difficult to rule out the possibility that the lack of a yolk testosterone response to aggression is due to a confounding factor, such as biased settlement or predation (e.g., Groothuis and Schwabl 2002).

Zebra finches (*Taeniopygia guttata*) are an ideal species to help experimentally elucidate how females allocate yolk testosterone in response to competition in a colonial species. They are colonial, yet are aggressive toward conspecific intruders while breeding (Evans 1970; Adkins-Regan and Robinson 1993; Zann 1996; Bolund et al. 2007) and are frequently used in maternal effect studies (e.g., Schwabl 1993; Gil et al. 1999; Adkins-Regan et al. 2013), thereby giving a context for studies manipulating yolk hormones. Furthermore, captive sexual behaviours, like aggression, have been shown to have no relationship with inbreeding coefficients (Forstmeier et al. 2004) and domestication has not created significantly different life-history strategies (Tschirren et al. 2009b). Hence, we experimentally tested the hypothesis that zebra finches, being a colonial species, would deviate from the pattern of increasing yolk testosterone in response to conspecific aggression as seen in more solitary species. We collected two clutches of eggs from zebra finch pairs laid in each of the following contexts: (1) untreated control clutches, and (2) experimental clutches laid during conspecific intrusions. This experiment was first

conducted as a pilot study in Spring 2014 and then again as a full study with an improved design in Winter 2015. For all females, we recorded the number of aggressive actions initiated by females prior to egg laying and measured yolk testosterone concentrations. If interspecific variation in yolk testosterone allocation has evolved to be adaptive for life-history traits influencing competition (e.g., coloniality), we predict that zebra finches will respond to increased aggression without increasing yolk testosterone.

METHODS

Animals and housing

Zebra finch pairs were individually housed in a room with a light dark cycle of 14:10h in standard cages (43 x 43 x 38 cm) with two perches, a nest-box, and burlap ribbon as nesting material. They were provided with a mixed seed diet, water, and cuttlebone *ad libitum*, and checked and refreshed daily. All animals were treated in accordance with University of Georgia's Institutional Animal Care and Use Committee (AUP #A2014-03-014-Y2-A0).

Pilot study

In March 2014, we conducted a pilot study to examine the influence of conspecific intrusions on the allocation of yolk testosterone. From ten zebra finch pairs, we monitored laying and collected the third egg of the clutch immediately after it was laid (i.e., control clutch). By collecting the third egg on the morning it is laid we have ensured that the hormones present in the egg are maternally derived, because this is prior to incubation when endogenous hormone production begins (Elf and Fivizzani 2002). Then, after allowing a two-month break in breeding, we exposed the same pairs to conspecific intrusions during egg-laying, and then collected the

third egg from each clutch (i.e., intrusion clutch). To do this, we introduced a novel female into the pairs' cage from 8 am to 5 pm each day to simulate a chronically intruding female. Intrusions started 3-7 days prior to clutch initiation, depending on how long it took females to begin laying, and continued until clutch completion. Despite the differences in clutch initiation, every female's third egg experienced at least six days of intrusions, which is approximately how long yolk deposition occurs prior to an egg being laid (Carey 1996). We performed 15 min observations during intrusions on four separate days from behind a blind located 2 m in front of the cage. During observations we recorded the frequency of four aggressive behaviours performed by the resident female toward the intruding female (Zann 1996): (1) bill-fencing, rapid jabbing of the closed bill at the head and bill of opponent; (2) supplanting, an individual is driven off a perch; (3) chasing, a displaced individual is followed; and (4) pecking, aggressive pecking at feathers and body regions other than the head.

Intrusion experiment

In December 2015, we expanded on the experimental design of the pilot study by (1) performing both treatments in first and second clutches to ensure our preliminary findings were not due to *between*-clutch variation, and (2) reducing the length of the conspecific intrusions to ensure previous findings were not due to the stress of lengthy intrusions. We collected the third egg from the first clutch of 16 zebra finch pairs each morning; eight pairs had no manipulation (i.e., control clutch) and eight pairs received conspecific intrusions (i.e., intrusion clutch). To simulate conspecific intrusions, we introduced a novel female into the pairs' cage for 1.5 hrs between 8 am and 12 pm and for 1.5 hrs between 12 pm and 5 pm; a length of time shown to increase aggression and yolk testosterone in collared flycatchers (*Ficedula albicollis*), a solitary

passerine (Hargitai et al. 2009). Intrusions started 1-10 days prior to clutch initiation, depending on how long females waited to lay their clutches, and continued until clutch completion. In January 2016, we swapped clutch treatments so that if a pair laid in control conditions for their first clutch they laid during intrusions for their second clutch and vice versa. We performed 15 min observations during control and intrusion clutches for each female on two separate days and aggressive behaviours toward mates (control clutches) and novel females (intrusions clutches) were measured as previously described.

Yolk testosterone

Yolk from collected eggs was homogenized and testosterone was extracted with a double ether extraction followed by liquid column chromatography (Schwabl 1993). Briefly, 50 mg of yolk was weighed and vortexed with 1000 μl of deionised water. Next, 3 ml of petroleum:diethyl ether (30:70 vol/vol) was added, the mixture was vortexed for 30 s and was allowed to settle for 20 min. Samples were then snap frozen and the supernatant was poured off and dried. The sample was reconstituted in 1.0 ml 10% ethyl acetate in isooctane and steroids were separated using celite column chromatography. Testosterone was eluted in 20% ethyl acetate in isooctane. Testosterone was quantified using a standard competitive-binding radioimmunoassay in a single assay per experiment (using anti-testosterone from MP Biomedicals, Solon, OH; Wingfield and Farner 1975). Recoveries averaged 79% and intra-assay variation averaged 4.7%.

Statistical analyses

All statistical analyses were performed in R (R Development Core Team 2013). We initially tested for seasonal differences between data collected in Spring 2014 and Winter 2015 to

ensure they were comparable using a linear mixed effects model with yolk testosterone or female aggression toward a novel intruder as the dependent variables and season (spring or winter) as the fixed effect with female ID as the random effect. For the pilot study in Spring 2014, we first tested how consistent individual aggressive behaviours were across the four observations as the intra-class correlation coefficient (Lessells and Boag 1987). We used a paired t-test to determine if females changed yolk testosterone concentration when eggs were laid during control or intrusion conditions. We also performed a linear regression to determine if yolk testosterone allocated during intrusions was related to (1) number of aggressive interactions, or (2) number of days they received intrusions prior to egg laying.

For the intrusion experiment in Winter 2015, we used linear mixed effects models with (1) yolk testosterone and (2) average number of aggressive actions as the dependent variable. In each model, treatment (control or intrusion), clutch number (1 or 2), and their interaction were included as fixed effects with female ID as the random effect. If the interaction term was not significant it was removed from the model. We ran a third linear mixed effects model to determine if average number of aggressive actions was related to yolk testosterone concentrations with female ID as the random effect during both control and intrusion clutches. We also performed a linear regression to determine if yolk testosterone allocated during intrusion clutches was related to the number of days females received intrusions prior to egg laying.

RESULTS

Despite the pilot study and intrusion experiment being performed in the spring and winter, respectively, we saw no effect of seasonality on egg parameters or female behaviours. Yolk testosterone in Spring 2014 and Winter 2015 did not differ for control (β = 7.23 +/- 9.86,

$F_{1,3}$ = 0.54, P = 0.52) or intrusion conditions (β = 5.51 +/- 3.02, $F_{1,3}$ = 3.14, P = 0.17). In a larger dataset of all breeding pairs, there was also no difference in egg mass across the four seasons ($F_{3,343}$ = 1.77, P = 0.15; Bentz and Navara, *unpubl.*). Aggressive behaviours by resident females toward a novel intruder also did not significantly differ between Spring 2014 and Winter 2015 (β = -1.38 +/- 0.97, $F_{1,3}$ = 2.05, P = 0.25), suggesting there were no seasonal effects.

Pilot study

In our pilot study, resident females on average performed 4.36 (+/- 1.13 SE, 0.75-13.5) aggressive actions toward an intruding female during a 15 min observation. Aggressive behaviours were also highly consistent (intra-class coefficient = 0.72; 0.48-0.95 CI), indicating that intruding females elicited consistent aggressive behaviour from the resident female. Average yolk testosterone was 22.98 pg/mg (+/- 2.37 SE), within the range for this species (Gil et al. 2004). Females did not significantly change yolk testosterone in eggs laid during intrusions (22.77 pg/mg +/- 3.61 SE) compared to control eggs (23.18 pg/mg +/- 3.27 SE, t_9 = 0.15, P = 0.88). The concentration of yolk testosterone laid in intrusion eggs was not significantly related to the average number of aggressive actions a female performed (β = -0.69 +/- 1.10 SE, $F_{1,8}$ = 0.40, P = 0.55) nor was it significantly influenced by the number of days females received intrusions (β = -1.48 +/- 2.80 SE, $F_{1,8}$ = 0.28, P = 0.61).

Intrusion experiment

The results of our full experiment corroborated the results of the pilot study. The number of aggressive actions was moderately consistent for females in intrusion contexts (intra-class coefficient = 0.56; 0.22-0.90 CI), again indicating that intruding females elicited consistent

behaviours from the resident female. Yolk testosterone did not significantly differ between control (22.04 pg/ml +/- 1.59 SE) or intrusion (25.80 pg/ml +/- 2.12 SE) conditions ($t_{1,12} = 1.82$, $P = 0.09$; Figure 3.1a) or between first (26.27 pg/ml +/- 2.03 SE) or second (22.36 pg/ml +/- 1.77 SE) clutches ($t_{1,12} = 0.65$, $P = 0.53$; Figure 3.1a). However, the number of aggressive interactions was significantly greater during intrusion (3.07 +/- 0.55 SE) than control (0.38 +/- 0.13 SE) treatments ($t_{1,14} = 4.81$, $P < 0.01$; Figure 3.1b). The level of aggressive behaviour was unrelated to whether the intrusion treatment was given first or second (β = -0.50 +/- 0.52 SE, $t_{1,14} = -0.96$, $P = 0.35$; Figure 3.1b). For both analyses, the interaction between treatment and clutch was not significant (all $P > 0.17$) and was removed. The number of aggressive actions performed by a female was not significantly related to the concentration of yolk testosterone ($\beta = 0.07$ +/- 0.53 SE, $t_{1,13} = 0.12$, $P = 0.90$). The concentration of yolk testosterone laid in intrusion eggs was also not significantly influenced by the number of days females received intrusions ($\beta = 0.27$ +/- 0.38 SE, $F_{1,13} = 0.48$, $P = 0.50$).

DISCUSSION

Females did not deposit higher concentrations of yolk testosterone when exposed to conspecific intrusions compared to control conditions. This occurred despite females performing significantly more aggressive actions during intrusions, levels of which were high compared to previous studies examining aggression in zebra finch females (Adkins-Regan and Robinson 1993). Our findings contradict the numerous studies showing a positive relationship between yolk testosterone and conspecific aggression (von Engelhardt and Groothuis 2011). Yet, when viewed within a life-history context, these experimental data agree with other largely correlative

studies conducted in colonial species that also fail to show a positive relationship between yolk testosterone and aggression (Bentz et al. 2016a).

The increase in yolk testosterone typically seen in response to aggressive interactions is often explained as a by-product of higher circulating testosterone in females responding to aggression (Groothuis et al. 2005). However, individuals from colonial species consistently have higher circulating plasma testosterone (Møller et al. 2005) and do respond to competition with aggression (Alexander 1974; Birkhead 1978; Poot et al. 2012), but do not deposit more yolk testosterone on average than solitary species (Gil et al. 2007). The relationship between female plasma and yolk hormones has yet to be conclusively described, even in non-colonial species, with studies showing both positive (Schwabl 1996; Jawor et al. 2007) and negative (Mazuc et al. 2003; Navara et al. 2006) relationships. It is plausible that plasma and yolk hormone allocation can be regulated independently (Hackl et al. 2003; Moore and Johnston 2008; Okuliarova et al. 2011; Egbert et al. 2013). If this is the case, it would allow selection to shape yolk testosterone responses independent of plasma or behavioural responses to aggressive interactions, which could explain the uncoupling observed in colonial species and may even be preferable given the changing adaptive value of offspring phenotype with life-history strategy.

Individuals from colonial species have more frequent conspecific interactions (Birkhead 1978; Klatt et al. 2004; Sachs et al. 2007) and a greater risk of pathogen infection (Brown and Brown 2004a,b) compared to solitary species. Thus, enhanced yolk testosterone, which some studies suggest causes faster development (e.g., Schwabl 1993; Lipar and Ketterson 2000), more aggressive behaviours (e.g., Strasser and Schwabl 2004; Eising et al. 2006; Partecke and Schwabl 2008), and decreased immune function (e.g., Navara et al. 2005; Rukowska et al. 2007; Sandell et al. 2009, but see Tobler et al. 2010), may not benefit colonial species when living in

higher densities. Indeed, colonial species tend to favour a reduction in intensity of aggression compared to less colonial species, for example, by colonial species having a larger repertoire of appeasement signals (Birkhead 1978) or by increasing attack latency (Pruitt et al. 2012). Therefore, because zebra finches are highly colonial (Zann 1996), they may not change yolk testosterone with increasing conspecific interactions because it would be maladaptive for the purposes of group-living to create more aggressive, faster growing, pathogen susceptible offspring. In support of this, zebra finches raised in larger groups during adolescence more easily integrate into new groups as adults (Ruploh et al. 2013) and are less aggressive (Ruploh et al. 2012), as opposed to those raised in solitude. This suggests decreasing the severity of aggression is a potential adaptation to group-living and preventing elevations of yolk testosterone may facilitate less-aggressive phenotypes.

The fitness consequences of yolk testosterone are unclear. There is mixed support for yolk testosterone's effect on early survival (von Engelhardt and Groothuis 2011). However, because zebra finches are an altricial species, meaning their interaction with the maternal environment as nestlings is limited, they may instead experience fitness consequences associated with exposure to high levels of yolk testosterone as adults. In particular, the effects of yolk testosterone on aggressive behaviours last into adulthood (Strasser and Schwabl 2004; Eising et al. 2006; Partecke and Schwabl 2008). Nevertheless, for selection to occur on adult offspring, those offspring must remain in or select a similar habitat to that of the maternal competitive environment that elicited the maternal effect. There is some evidence that habitat choice has a heritable component (Brown and Brown 2000) and zebra finches have shown a preference for habitats similar to that of their natal habitat (Sargent 1965). Indeed, a wide range of taxa have demonstrated a post-dispersal habitat preference for that which is similar to what they

experienced early in life (Davis and Stamps 2004). Therefore, selection against increasing yolk testosterone in response to competition could possibly occur in the adult stage if more aggressive offspring, from high yolk testosterone eggs, are not as successful in more competitive environments when they are a colonial species.

Conclusions

Our findings in zebra finches, in conjunction with the primarily correlative findings in other colonial species, suggest that colonial species do not increase the allocation of maternal testosterone to egg yolks in response to aggressive interactions. This highlights the importance of testing maternal effect hypotheses in many different species while also considering their life-history traits when interpreting the results.

FIGURES

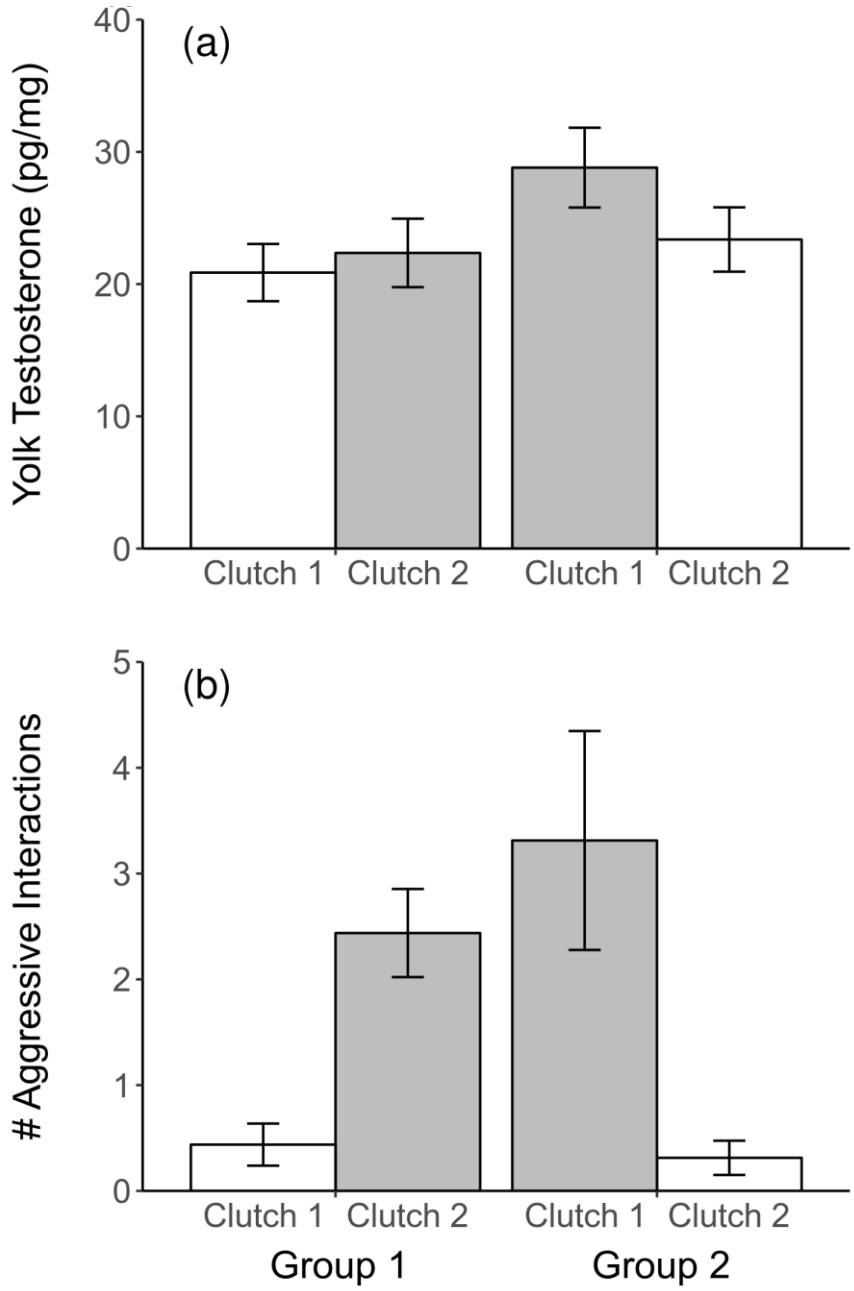

Figure 3.1. Yolk testosterone concentration (a) and number of aggressive actions initiated (b) by females breeding in control (white bars) or conspecific intrusion (grey bars) contexts during first or second clutches during the Winter 2015 intrusion experiment. Group 1 females bred in a control context first (N = 8) and then the intrusion context (N = 8), and group 2 females bred in the intrusion context first (N = 8) and then the control context (N = 8). Error bars represent SE.

CHAPTER 4

RELATIONSHIP BETWEEN MATERNAL ENVIRONMENT AND DNA METHYLATION PATTERNS OF ESTROGEN RECEPTOR ALPHA IN WILD EASTERN BLUEBIRD (*SIALIA SIALIS*) NESTLINGS: A PILOT STUDY.[A]

[A] Bentz, A.B., Sirman, A.E., Wada, H., Navara, K.J. and Hood, W.R. 2016. Relationship between maternal environment and DNA methylation patterns of estrogen receptor alpha in wild Eastern Bluebird (*Sialia sialis*) nestlings: a pilot study. Ecol. Evol. 6: 4741-4752. Reprinted here with permission of publisher.

ABSTRACT

There is mounting evidence that, across taxa, females breeding in competitive environments tend to allocate more testosterone to their offspring prenatally and these offspring typically have more aggressive and faster-growing phenotypes. To date, no study has determined the mechanisms mediating this maternal effect's influence on offspring phenotype. However, levels of estrogen receptor alpha (ERα) gene expression are linked to differences in early growth and aggression; thus, maternal hormones may alter gene regulation, perhaps via DNA methylation, of ERα in offspring during prenatal development. I performed a pilot study to examine natural variation in testosterone allocation to offspring through egg yolks in wild Eastern Bluebirds (*Sialia sialis*) in varying breeding densities and percent DNA methylation of CG dinucleotides in the ERα promoter in offspring brain regions associated with growth and behavior. Yolk testosterone concentration was positively correlated with breeding density, nestling growth rate, and percent DNA methylation of one out of five investigated CpG sites (site 3) in the diencephalon ERα promoter, but none in the telencephalon (n = 10). Percent DNA methylation of diencephalon CpG site 3 was positively correlated with growth rate. These data suggest a possible role for epigenetics in mediating the effects of the maternal environment on offspring phenotype. Experimentally examining this mechanism with a larger sample size in future studies may help elucidate a prominent way in which animals respond to their environment. Further, by determining the mechanisms that mediate maternal effects, we can begin to understand the potential for the heritability of these mechanisms and the impact that maternal effects are capable of producing at an evolutionary scale.

Key words: maternal effect, yolk testosterone, breeding density, growth rate, diencephalon

INTRODUCTION

The social environment experienced by females can have formative impacts not only on her survival and reproductive success, but also on that of her offspring. One way in which this occurs is through hormone-mediated maternal effects (i.e., embryonic exposure to environmentally elicited maternal hormones that modify offspring phenotype; Groothuis et al. 2005). Several studies have shown that females breeding in high density and/or increased social interactions allocate more testosterone to their young prenatally (Whittingham and Schwabl 2001; Mazuc et al. 2003; Pilz and Smith 2004; Dloniak et al. 2006; Hargitai et al. 2009; Bentz et al. 2013; but see von Engelhardt and Groothuis 2011); these offspring then display more-competitive traits like faster post-natal growth during the early phase of rapid mass gain (Schwabl 1996; Eising et al. 2001; Pilz et al. 2004; Navara et al. 2005; Navara et al. 2006a; Cucco et al. 2008; Bentz et al. 2013; but see Gorman and Williams 2005) and increased aggressive, competitive behaviors (Strasser and Schwabl 2004; Eising et al. 2006; Dloniak et al. 2006; Partecke and Schwabl 2008; Müller et al. 2009). Maternal effects are potentially a way by which offspring can match their phenotype to current environmental conditions and the adaptive significance has been of considerable interest to evolutionary biologists (Mousseau and Fox 1998; Mcadam et al. 2002; Räsänen and Kruuk 2007). However, the link between maternal hormones and offspring phenotype is not well understood. Without knowing the mechanisms that mediate maternal effects, we cannot begin to fully understand the heritability of this phenomenon and how it fits into a larger evolutionary framework.

Few studies have attempted to test mechanisms by which yolk testosterone influences offspring phenotype. One hypothesis is that yolk testosterone enhances growth by increasing begging rates (Schwabl 1996), but there is conflicting support (Pilz et al. 2004; Müller et al.

2012). Pfannkuche et al. (2011) found that offspring exposed to higher yolk testosterone concentrations had lower circulating testosterone and androgen receptor (AR) mRNA expression in whole brain tissue, suggesting that yolk testosterone may influence offspring phenotype by mediating changes in levels of ARs. However, testosterone does not restrictively act through ARs, but can also be converted to estrogen via aromatase and bind to the estrogen receptor (ER; Groothuis and Schwabl 2008). For example, Hegyi and Schwabl (2010) showed that when dihydrotestosterone (i.e., an unaromatizable metabolite of testosterone that only utilizes the AR) was injected into Japanese quail (*Cotrunix japonica*) eggs, offspring growth was not affected. In Müller et al. (2005), egg injections of an AR antagonist also did not decrease growth in male Black-headed Gulls (*Larus ridibundus*), a species whose growth was positively affected by yolk testosterone in a previous study (Eising et al. 2001). Thus, testosterone may act through estrogen and its ER, which are known to influence growth hormone (GH), a regulator of early growth (Meinhardt and Ho 2006; Addison and Rissman 2012). The hypothalamus (a large component of the diencephalon) expresses growth hormone-releasing hormone (GHRH), a regulator of GH (Harvey 1985) that has estrogen-responsive elements in its promoter (Petersenn et al. 1998), and both GH and GHRH are co-expressed with ERα in the hypothalamus (Kamegai et al. 2001; Addison and Rissman 2012). Furthermore, estrogens are implicated in other phenotypic changes associated with yolk testosterone, such as aggressive behaviors (Soma 2006). Aggressive phenotype is correlated with ERα mRNA expression in the diencephalon and telencephalon (Filby et al. 2010; Rosvall et al. 2012), and both aromatase inhibitors and ERα antagonists decrease aggressive behaviors (Walters and Harding 1988; Schlinger and Callard 1990). Songbirds express ERα mRNA in the diencephalon and posterior telencephalon during late

embryonic development irrespective of sex (Perlman and Arnold 2003). Thus, yolk testosterone may alter ERα expression in the diencephalon and telencephalon to cause phenotypic changes.

Epigenetic modifications have become promising candidates for explaining how environmental stimuli can influence early development. Epigenetic effects create stable changes in gene expression without altering DNA sequence; for example, adding methyl groups to cytosines at CG dinucleotides (i.e., CpG sites) in gene promoters typically suppresses gene expression (Holliday 1994). Organisms are most susceptible to epigenetic modifications in early development (Vickaryous and Whitelaw 2005). Most notably, sexual differentiation of the brain is thought to be due to endogenous prenatal steroid-induced alteration of steroid-receptor gene, primarily ERα, methylation (Schwarz et al. 2010) and histone acetylation (Matsuda et al. 2011). Moreover, it is during early development that animals are most dependent on maternal factors and it stands to reason that maternal effects could influence epigenetic patterns. Maternal influence in the early postnatal environment can affect DNA methylation status in offspring (Weaver et al. 2004; Murgatroyd et al. 2009), specifically, DNA methylation of the ERα (Champagne et al. 2006). Studies concerning prenatal epigenetic maternal effects often focus on the maladaptive effects of maternal exposure to pollutants, poor nutrition, or stress (Feil and Fraga 2012). However, animals are exposed to exogenous testosterone during prenatal development (Parsons 1970; von Engelhardt et al. 2009), yet few studies examine this effect. One study (Mori et al. 2010) did show a relationship between prenatal exposure to naturally occurring exogenous inputs of testosterone and decreased ERα DNA methylation and greater ERα mRNA expression in the hypothalamus along with increased aggression. This study supports the hypothesis that maternal social environment could affect offspring phenotype through prenatal testosterone-induced epigenetic modifications.

In the present pilot study, we investigated relationships between natural variation in the competitive environment experienced by Eastern Bluebirds (*Sialia sialia*) and their yolk testosterone allocation, offspring growth, and ERα DNA methylation in offspring brain tissue to help inform and promote future experimental studies. Eastern Bluebirds are obligate secondary cavity nesters and are limited by available cavities, causing intense competition for cavities in high breeding densities (Pinkowski 1976; Parren 1991; Gowaty and Plissner 1998). Because yolk testosterone concentrations have been linked to competitive environment and influence offspring growth rates and aggression in other species (von Engelhardt and Groothuis 2011), we hypothesized that (1) higher breeding densities would be positively correlated with testosterone allocation to egg yolks and (2) that exposure to high yolk testosterone concentrations would be related to faster growth rates in Eastern Bluebirds. Further, because previous work cited above indicates that testosterone may affect offspring phenotype via aromatization to estrogen, we further hypothesized that (3) higher yolk testosterone concentrations would be associated with decreased ERα DNA methylation in the diencephalon and posterior telencephalon in offspring and (4) patterns of ERα DNA methylation would negatively correlate with offspring growth rates.

METHODS

Study population

We monitored 142 nest boxes placed throughout Auburn, AL (32.5978^{o} N, 85.4808^{o} W) in 2011 from March through May. We were able to observe 26 Eastern Bluebird pairs and we collected the fourth egg from the first breeding attempt (n = 19; seven eggs were either unable to be collected prior to incubation or lost in processing). The fourth egg is representative of the entire clutch, because bluebirds display low within-clutch variation in yolk testosterone (Navara

et al. 2006b; Duckworth et al. 2015). We then measured body mass (± 0.01g) of nestlings from nests that successfully hatched (n = 25; one nest was lost to predation) on days two, five, eight, and 11 post-hatch, and fledging mass at 14 days post-hatch. Growth followed a sigmoidal pattern and reached a plateau around day 11. Therefore, the growth rate for each nestling was derived from the slope of a linear regression of nestling mass on days 2–11 post-hatch to ensure we captured the period of rapid, linear growth (for all nestlings: $r^2 > 0.80$). We sacrificed one randomly selected 14-day-old nestling from 17 different nests (six male and 11 female nestlings) for DNA methylation analyses. Brains were immediately dissected and post-fixed in buffered 4% paraformaldehyde for eight days at 4°C. Brains were then cryoprotected in 30% sucrose solution until they were fully penetrated by the cryoprotectant (~48 hrs) before they were frozen on ground dry ice and stored at -80°C. To dissect the diencephalon and posterior telencephalon, we discarded the cerebellum and performed a punch biopsy of the underlying diencephalon, acquiring primarily hypothalamic tissue, and collected the portion of the posterior telencephalon consisting of the nucleus taeniae of the amygdala according to the revised songbird brain atlas by Reiner et al. (2004). All procedures were conducted according to protocol #2011-1887 approved by the Auburn University Institutional Animal Care and Use Committee.

Breeding density was measured using Google Earth Pro to map the GPS location of all nest boxes at the field site and to create polygons encompassing each nest box territory. Polygon territories were defined as useable habitat (i.e., open meadows that have less than 50% tree cover) within a 300 m radius around each nest box as used in Duckworth et al. (2015). The area of each polygon was measured in hectares and the number of occupied nest boxes within each territory was counted to create a density measure of occupied nest boxes per hectare. We considered nest boxes within the territory occupied if a pair was present during the six days prior

to the focal pair laying their fourth egg, because yolk deposition can occur six days prior to an egg being laid (Navara et al. 2006b).

Yolk hormone analysis

Yolk testosterone was extracted from homogenized yolk samples with a double ether extraction followed by liquid column chromatography according to methods described by Schwabl (1993). Briefly, 50 mg of yolk was weighed and vortexed with 1000 µl of deionized water. Next, 3 ml of petroleum:diethyl ether (30:70 vol/vol) was added, the mixture was vortexed for 30 s and was allowed to settle for 20 min. Samples were then snap frozen and the supernatant was poured off and dried. The sample was reconstituted in 1 ml 10% ethyl acetate in isooctane and steroids were separated using celite column chromatography. Testosterone was eluted in 20% ethyl acetate in isooctane. Testosterone was quantified using standard competitive-binding radioimmunoassays using anti-testosterone (MP Biomedicals, Solon, OH) as described in Wingfield and Farner (1975). Anti-testosterone had a cross-reactivity of 100% with testosterone, 18.75% with dihydrotestosterone, 3% with androstanediol, and <1% with all other steroids. Average recoveries were 89% and average intra-assay variation was 8%.

Promoter identification

The Eastern Bluebird genome has not yet been sequenced and annotated. However, regions of gene regulation such as promoters are generally highly conserved across species (Carninci et al. 2006), so to identify a putative promoter region for the ERα gene in Eastern Bluebirds we used the most closely related songbird genome that has been fully sequenced as a starting point for comparison across species (i.e., Zebra Finch, *Taeniopygia guttata*; Warren et al.

2010). We identified 4000 bp of 5' flanking region and the 434 bp exon one of the Zebra Finch ERα gene using Ensembl (ENSTGUG00000011249) and compared sequence homology using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). We only compared sequences with at least 96% similarity that were also from the order Passeriformes and predicted to be part of the ERα gene; this included nine species: *Geospiza fortis*, *Corvus cornix*, *Corvus brachyrhynchos*, *Acanthisitta chloris*, *Serinus canaria*, *Manacus vitellinus*, *Ficedula albicollis*, *Zonotrichia albicollis*, and *Pseudopodoces humilis*. We then designed primers for the most highly conserved region. The forward primer (5'-ACCCAGACACACAAACATAC-3') and reverse primer (5'-GCAGTGAGCAAGGAACAT-3') were designed with PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA, USA).

DNA was extracted from homogenized posterior telencephalon brain tissue using a DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. PCR was performed in 50 µl which contained 1 µl of DNA template, 1 µl each of the forward and reverse primers, and 25 µl of PCR Master Mix, 2x (Promega, Madison, WI, USA). The PCR conditions were an initial denaturation of 94°C for 2 min then 35 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 30s with a final extension step of 72°C for 10 min. PCR products were visualized under ultraviolet light after 2% agarose gel electrophoresis. PCR products were purified using DNA Clean and Concentrator (Zymo Research, Irvine, CA, USA) and sequenced in both directions at the Auburn University Genomics and Sequencing Lab. Nucleotide sequences were read and assembled using the software Chromas Lite (Technelysium Pty. Ltd.). We identified potential transcription factor binding sites (TFBSs) on the putative Eastern Bluebird ERα promoter using MatInspector (Genomatix; Cartharius et al. 2005). We checked for corresponding TFBSs in the annotated Zebra Finch promoter. We included binding

sites of all TFBS that have been identified in brain tissue according to MatInspector (for a list of all potential TFBSs see Appendix B, Table S4.1).

DNA methylation analysis

We performed bisulfite-PCR on a 339 bp region of the 5' flanking region immediately upstream of exon one (i.e., putative promoter region) in both the diencephalon and posterior telencephalon. DNA extraction and bisulfite conversion were performed simultaneously with 0.1 mg of homogenized tissue using the EZ DNA methylation Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol for fixed tissue. The forward primer (5'-GAAAAATTAAAAGATTAGTAAGAATGAAGT-3') and reverse primer (5'-AAACAAAAAACATATCTACTTTCACT-3') for bisulfite-converted DNA were designed using Methyl Primer Express (Applied Biosystems). PCR was performed in 25 µl which contained 4 µl of bisulfite-converted DNA template, 1 µl each of the forward and reverse primers, and 12.5 µl of Zymo*Taq* DNA Polymerase (Zymo Research, Irvine, CA, USA). The PCR conditions were an initial denaturation of 95^{o}C for 10 min then 35 cycles of 95^{o}C for 30s, 52^{o}C for 30s, and 72^{o}C for 60s with a final extension step of 72^{o}C for 7 min. PCR products were visualized under ultraviolet light after 2% agarose gel electrophoresis. Due to low DNA concentrations, we used 1µl of PCR product as the template for a second round of PCR using the same primer pair and only 30 cycles. PCR products were visualized, purified, sequenced, and assembled as previously described. To determine the efficiency of the bisulfite conversion, we calculated conversion rate for each sample as the percent of cytosines not at a CpG site that were converted to thymine (Jiang et al. 2010). Percent DNA methylation for each CpG site was calculated as the peak height of cytosine divided by the sum of the peak height for cytosine and

thymine (Jiang et al. 2010). Calculating percent DNA methylation using direct bisulfite-PCR sequencing has been shown to produce comparable results to that of pyrosequencing and bisulfite-cloning sequencing (Jiang et al. 2010).

Statistical analyses

For hypotheses one and two, we first performed a linear regression to determine if variation in yolk testosterone concentration could be explained by breeding density. Next, we used a linear mixed effects model to determine if either growth rate or fledging mass at day 14 were correlated with yolk testosterone concentration while controlling for offspring sex and brood size. For the mixed effects model, nest box ID was the random effect to account for the fact that some nestlings came from the same brood, and this model was fit with restricted maximum likelihood using the *nlme* package in R (Pinheiro et al. 2015).

To address hypotheses three and four, we performed separate linear regressions for each CpG site in the diencephalon and telencephalon to test 1) if yolk testosterone concentration was correlated with percent DNA methylation of each CpG site (n = 10; of the 17 brain samples, only 10 were from nests in which a yolk sample had been collected) and 2) if percent DNA methylation of CpG sites was related to growth phenotype while accounting for brood size and offspring sex (n = 17 for fledging mass analysis and n = 16 for growth rate analysis; one nest was only visited twice and growth rate could not be confidently calculated). We chose not to use *P*-value adjustments to correct for multiple testing when examining percent DNA methylation of the five CpG sites within each brain region, because there were only five comparisons and our small sample size already makes us conservative with type I error fixed at $\alpha = 0.05$ and particularly susceptible to type II error, which *P* -value corrections would exacerbate (Rothman

1990; Johnson 1999). Additionally, we tested all model residuals for outliers using the Grubbs' outlier test (Grubbs 1950) with the *outliers* package in R (Komsta 2011), because small sample sizes, such as ours, are sensitive to observations that have high leverage or are too influential making ordinary least squares regression methods inappropriate. When outliers were detected, we used a robust linear regression to calculate estimates that are not as strongly affected by outliers (Rousseeuw and Leroy 1987). Specifically, we employed an SMDM-type regression estimator that performs well with small sample sizes (Koller and Stahel 2011) using the *robustbase* package in R (Rousseeuw et al. 2015). We also performed correlations with percent DNA methylation between and within CpG sites in the diencephalon and telencephalon to look for inter- and intra-relationships. All statistical analyses were performed with R version 3.0.1 (R Development Core Team 2013). All means are followed by the standard error.

RESULTS

Breeding density, yolk testosteorne, and offspring growth

On average, there were 4.7 (± 0.4; 1-11) nest boxes in each territory, of which 78.8 % (± 3.6; 40-100 %) were occupied. The number of occupied nest boxes per hectare was a significant and positive predictor of yolk testosterone (β = 28.98 ± 12.99, r^2 = 0.23, $F_{1,17}$ = 4.98, P = 0.04; Figure 4.1). Growth rate was significantly and positively correlated with yolk testosterone (β = 0.01 ± 0.01, $F_{1,14}$ = 7.55, P = 0.02) and negatively correlated with brood size (β = -0.11 ± 0.04, $F_{1,14}$ = 6.20, P = 0.03), but nestling sex was not a significant predictor of nestling growth rate (P = 0.41). Neither yolk testosterone (P = 0.52), sex (P = 0.16), or brood size (P = 0.59) were significantly correlated with fledging mass at day 14.

Promoter identification

We compared the 5' flanking region and exon one of the ERα gene across 10 avian species and found that the most highly conserved region of the 4,434 bp sequence was exon one and 216 bp of the immediately upstream 5' flanking region. This highly conserved region also coincided with the promoter region of the Zebra Finch ERα gene identified by ElDorado, the Genomatix genome annotation (Cartharius et al. 2005). Using primers designed to detect this region, we obtained a 656 bp fragment from Eastern Bluebird telencephalon tissue which we aligned with the Zebra Finch genome using BLAT (USCS Genome Browser) and found that it had a 99.6% identity with the promoter of the Zebra Finch ERα gene. Using the 656 bp region as a template, we aimed to determine the DNA methylation pattern of the most highly conserved region and likely promoter (i.e., 216 bp upstream of exon one) of the Eastern Bluebird ERα gene.

Yolk testosterone and DNA methylation

Bisulfite-PCR produced a 339 bp fragment in the putative promoter region of the Eastern Bluebird ERα gene, in which five CpG sites and all potential TFBSs specific to brain tissue were identified (Figure 4.2; for a list of all potential TFBSs see Appendix B, Table S4.1). Our bisulfite conversion rate was 91.48% (+/- 0.87) in the diencephalon and 93.20% (+/- 0.83) in the telencephalon. Average percent DNA methylation of CpG sites within the diencephalon was as follows: CpG site 1 = 68.91% (+/- 4.36), CpG site 2 = 56.03% (+/- 2.98), CpG site 3 = 58.38% (+/- 3.29), CpG site 4 = 96.44% (+/- 1.42), and CpG site 5 = 81.46% (+/- 1.75). Average percent DNA methylation of CpG sites within the telencephalon was as follows: CpG site 1 = 65.93% (+/- 4.16), CpG site 2 = 51.62% (+/- 3.92), CpG site 3 = 49.59% (+/- 3.73), CpG site 4 = 97.73% (+/- 0.82), and CpG site 5 = 74.57% (+/- 3.97). Contrary to what we hypothesized, yolk

testosterone concentration was significantly and positively correlated with percent DNA methylation of CpG site 3 in the ERα in the diencephalon (Figure 4.3a), but not with any other CpG sites (Table 4.1). Percent DNA methylation of ERα was not strongly correlated between CpG sites either within or between brain regions in an individual (all $r < 0.63$).

Nestling growth and DNA methylation

We tested if percent methylation of CpG site 3 in the diencephalon (i.e., the only CpG site that was correlated with yolk testosterone concentration) could explain variation in growth rate. Grubbs' outlier test indicated that an outlier was present in the residuals ($G = 3.03$, $P < 0.01$), thus, we used robust linear regression methods and found that growth rate was significantly and positively correlated with percent methylation of CpG site 3 in the diencephalon (robust regression: $\beta = 0.01 \pm 0.002$, $t_{3,12} = 2.71$, $P = 0.02$; linear regression: $\beta = 0.01 \pm 0.004$, $t_{3,12} = 1.40$, $P = 0.19$; Figure 4.3b). Growth rate was also significantly, negatively correlated with brood size (robust regression: $\beta = -0.12 \pm 0.04$, $t_{3,12} = -3.11$, $P = 0.01$; linear regression: $\beta = -0.19 \pm 0.07$, $t_{3,12} = -2.57$, $P = 0.02$), but not offspring sex (robust regression: $P = 0.32$; linear regression: $P = 0.63$). Fledging mass at day 14 was not correlated with CpG site 3 in the diencephalon; there was an outlier ($G = 2.49$; $P = 0.05$) but this did not change the significance of the outcome (robust regression: $P = 0.96$; linear regression: 0.90).

DISCUSSION

Our findings agree with previous studies showing that yolk testosterone is positively correlated with breeding density and offspring postnatal growth (see Introduction). Our preliminary data also suggest that ERα DNA methylation in the diencephalon may play a role in

linking maternal environment and growth rate, although our data should be interpreted cautiously given our limited sample size. We found a positive correlative relationship between yolk testosterone concentration and DNA methylation of CpG site 3 in the ERα promoter in the diencephalon. Furthermore, ERα CpG site 3 percent DNA methylation in the diencephalon was positively related to growth rate. Our analysis of the putative promoter region indicated that CpG site 3 was in close proximity to several potential TFBS, one of which was a TATA box (i.e., a strong initiator of transcription; Carninci et al. 2006; Kitazawa and Kitazawa 2007), signifying that it could have important implications for transcriptional control. While we did not measure ERα mRNA expression, Fürst et al. (2012) showed that it is possible for its expression to be decreased by greater methylation of a single CpG site. Regardless, future experimental manipulations should be performed to investigate the directionality and causality of the patterns found in our correlative study.

There are several potential explanations for why we found correlative relationships between percent DNA methylation at CpG 3 in the diencephalon, yolk testosterone, and growth rate. One of the more intriguing explanations to explore in future research is the relationship between ERα, GH, and growth. One of the main factors known to regulate food intake and growth in birds is GH (Buntin and Figge 1988). We had predicted decreased methylation of ERα, but estrogen can have both positive (Hassan et al. 2001; Yan et al. 2004) and negative effects (Lam et al. 1996; Petersenn et al. 1998) on GH, likely due to variation in estrogen concentration and GH's ability to autoregulate (Tennenbaum 1980; Bagamasbad and Denver 2011). For example, Childs et al. (2005) showed that low concentrations of estrogen stimulate more GH cells to display GHRH binding sites. In one of the few avian studies, Hall et al. (1984) incubated chicken pituitary glands in hypothalamic extract and measured GH secretion with and without

estrogen priming and found that estrogen-primed pituitaries were less sensitive to GH releasing activity. Furthermore, the effects of estrogen on GH are blocked if an ERα antagonist is administered (Avtanski et al. 2014), providing evidence that estrogen regulates GH via ERα. Therefore, lower levels of estrogen and ERα may increase cellular sensitivity to hypothalamic GHRH during postnatal growth and if ERα expression was indeed lowered in our study then this could help explain the positive correlation with growth rate.

Additionally, concentrations of GH in birds peak during the period of rapid, early growth and then decline, remaining low throughout adulthood (Scanes and Balthazart 1981; Scanes et al. 1992; Schew et al. 1996). This may explain why we found a relationship between percent DNA methylation and early growth rate, but not fledging mass, which was measured approximately 3 days after peak mass was reached. Thus, the relationship we found between ERα DNA methylation in the diencephalon and growth could be related to the interaction between ERα and GHRH in this brain region. However, our study was purely correlative and further experimentation is needed to fully test this idea.

The patterns we found between yolk testosterone, growth, and percent ERα DNA methylation in the diencephalon were not found in the telencephalon, and it is not clear why only the diencephalon would show these relationships. It may simply be that we lacked the power to detect these relationships and future studies should not be deterred from investigating this brain region in the context of yolk testosterone. However, another possibility is that, in birds, the diencephalon has greater levels of aromatase activity than the telencephalon (Balthazart et al. 1990) and testosterone can suppress aromatase activity (Bagamasbad and Denver 2011). Exposure to high testosterone concentrations early in development may lead to a permanent downregulation of estrogenic activity in the diencephalon, but not the telencephalon.

Furthermore, the telencephalon is implicated in social behaviors but not growth (Goodson 2005), supporting a lack of a relationship between ERα DNA methylation in the telencephalon and growth.

The other well-studied effect of yolk testosterone on offspring phenotype is an increase in aggressive, competitive behaviors (see Introduction). We were unable to measure aggression due to the fact Eastern Bluebirds are altricial and do not express explicit aggressive behaviors until after fledging. However, our findings may still be tentatively applied to the effects of yolk testosterone on aggression. Rosvall et al. (2012) showed that more aggressive Dark-eyed Juncos (*Junco hyemalis*) had greater expression of ERα in the telencephalon but lower expression in the hypothalamus. This mirrors our findings, in that we found greater DNA methylation in the diencephalon, which is suggestive of lower expression in individuals exposed to more yolk testosterone (Fürst et al. 2012).

An alternative explanation for our findings could be that they are an artifact of our small sample size or other unmeasured factors. We chose not to use *P*-value adjustments when investigating relationships between percent methylation at the five CpG sites and yolk testosterone in each brain region, because of the trade-off between type I and type II error it would require. Small sample sizes, such as ours, are particularly prone to type II errors (Johnson 1999), and adjusting *P*-values would decrease the possibility of type I errors at the expense of type II errors (Rothman 1990). In a pilot study meant to prompt future research, we felt that the cost of a false negative was much greater than that of a false positive. Therefore, the significant relationships we found with percent methylation at CpG site 3 in the diencephalon could be a result of type I error; however, it is more likely that there are more biologically significant relationships than we were able to statistically detect. There are also other components of yolk

that are affected by breeding density and/or social interactions that could affect growth that we did not measure in this study. For example, lesser black-backed gulls (*Larus fuscus*) increase yolk carotenoids with frequency of social interactions (Verboven et al. 2005) and black-headed gulls (*Larus ridibundus*) increase yolk antibodies with breeding density (Müller et al. 2004); however, these relationships have yet to be found in passerines (Hargitai et al. 2009; Safran et al. 2010; Remeš et al. 2011). Birds nesting in higher densities also tend to deposit less corticosterone in their egg yolks (Love et al. 2008; but see Bentz et al. 2013). Nevertheless, yolk androgens are the best-studied effect of breeding density and/or social interactions (von Engelhardt and Groothuis 2011) and, despite the correlative nature of our study, the best candidate for the effects we measured.

Conclusions

Hormone-mediated maternal effects potentially cause adaptive changes in offspring phenotype and, while past studies have examined what causes females to vary prenatal hormones and what offspring phenotypes arise as a consequence, it is still unclear how maternal hormones exert their influence. The preliminary data presented herein shed light on the potential mechanisms that mediate the effect of environmentally elicited maternal testosterone on offspring phenotype and suggest avenues for future studies. Thus far, few studies (see Müller et al. 2005) have injected eggs with an AR antagonist and measured growth. Future studies could administer AR or ERα antagonists or aromatase inhibitors into yolk along with testosterone to directly test the hypothesis that yolk testosterone acts through ERα. Also, because we sacrificed nestlings on day 14, our correlative study is unable to separate whether percent DNA methylation was prenatally programmed and influenced growth or if postnatal growth

programmed percent DNA methylation. While most epigenetic programming occurs prenatally (Vickaryous and Whitelaw 2005), postnatal experiences have been shown to influence DNA methylation patterns (Weaver et al. 2004; Champagne et al. 2006; Murgatroyd et al. 2009). Thus, while our findings do give a natural context, it is important for future studies to experimentally test these ideas while incorporating a cross-foster design. Finally, further analysis of the structure of the ERα promoter in passerines is necessary to provide insights into the mechanisms that regulate the expression of ERα and to provide tools to investigate the relevance of these mechanisms to maternal effects. Ultimately, by determining the mechanism by which maternal effects influence offspring phenotype, we can begin to understand the potential for the heritability of these mechanisms and the impact that maternal effects are capable of producing at an evolutionary scale.

Data accessibility

All data associated with this manuscript are archived in GenBank (accession number KT852372) and Dryad (doi:10.5061/dryad.4351q).

TABLES AND FIGURES

Table 4.1. Linear regression analyses of the percent DNA methyaltion of each CpG site in the putative promoter region of estrogen receptor alpha in 14 day old Eastern Bluebird offspring in the diencephalon and telencephalon with yolk testosteorne concentration as the predictor variable. Only one significant outlier was detected using Grubbs' outlier test in the residuals of the regression between percent DNA methylation of telencephalon CpG site 4 and yolk testosterone (G = 2.41, $P < 0.01$); however, whether linear regression ($P = 0.72$) or robust regression ($P = 0.91$) were used the signifcane of the outcome did not change, so the linear regression results are presented in the table.

Brain Region	CpG	β (SE)	DF	F	P
Diencephalon	1	0.16 (1.40)	1, 8	0.01	0.91
	2	0.01 (0.46)	1, 8	<0.01	0.99
	3	1.89 (0.82)	1, 8	5.34	**0.049**
	4	-0.20 (0.38)	1, 8	0.28	0.61
	5	-0.11 (0.74)	1, 8	0.02	0.88
Telencephalon	1	-0.65 (1.47)	1, 8	0.20	0.67
	2	-0.74 (1.60)	1, 8	0.21	0.66
	3	-1.71 (1.11)	1, 8	2.37	0.16
	4	0.13 (0.34)	1, 8	0.14	0.72
	5	-0.14 (1.54)	1, 8	0.01	0.93

Bold values represent $P<0.05$.

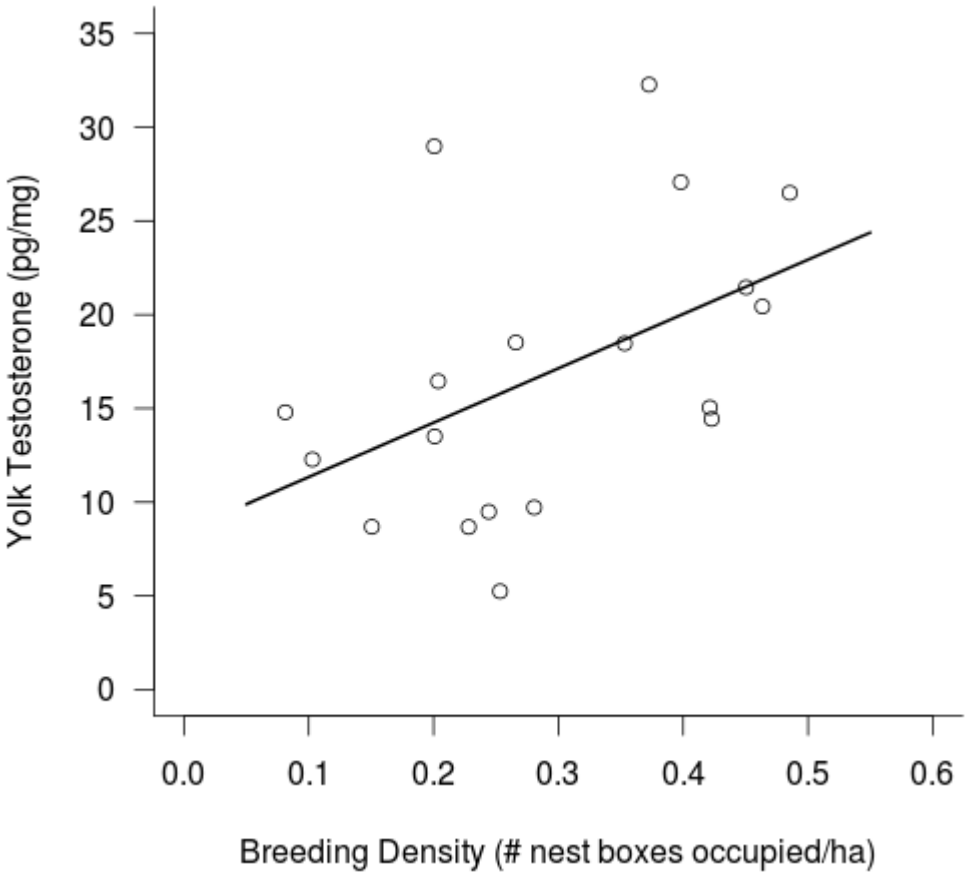

Figure 4.1. Correlation between Eastern Bluebird breeding densities (i.e., the number of occupied nest boxes per area of useable habitat within a 300 m radius of each box) and yolk testosterone concentrations in the fourth egg.

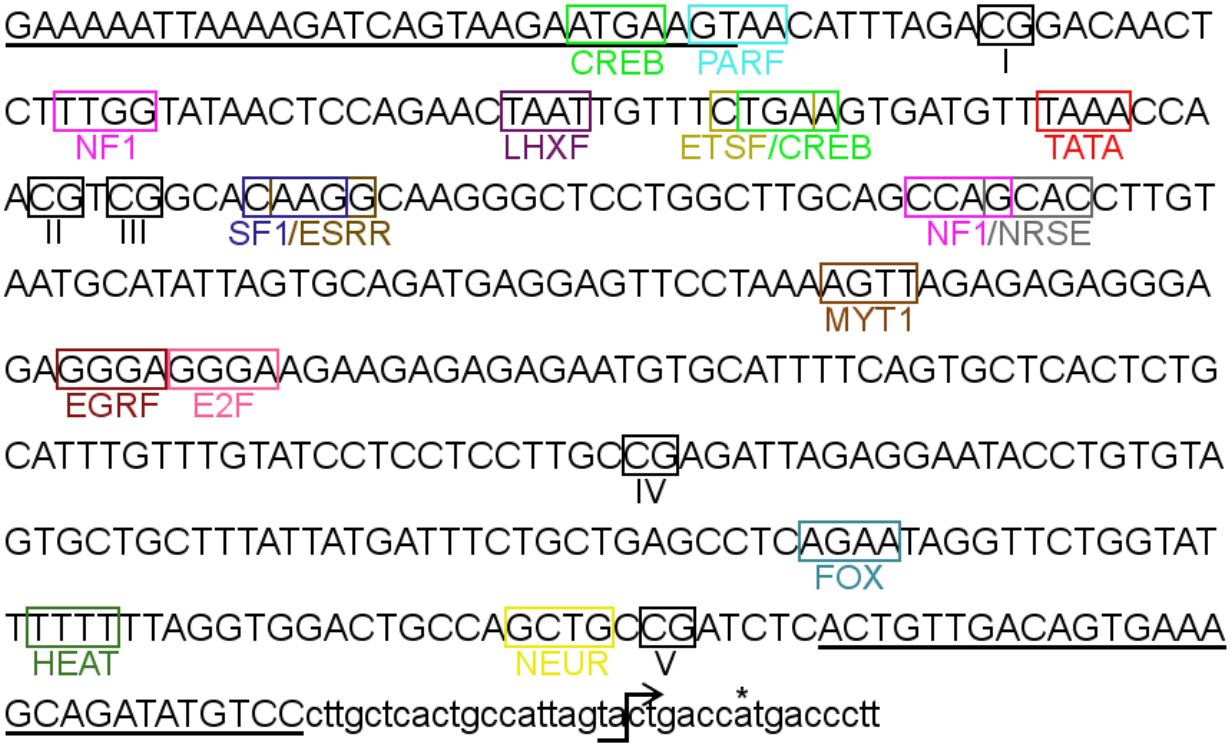

Figure 4.2. Sequence for the putative promoter region of estrogen receptor alpha in the Eastern Bluebird. The core conserved sequences (four nucleotides) for transcription factor binding sites and CpG sites (labeled with roman numerals I-V) are indicated in colored and black boxes, respectively. Upper case letters were sequenced from Eastern Bluebird brain tissue and lower case letters are from Zebra Finches (ENSTGUG00000011249) so that the potential transcription start site could be shown. The transcription start site is indicated with an arrow. A potential translation initiation codon, ATG, is indicated with an asterisk. The sequences of the primer pairs used for bisulfite-PCR are underlined. CREB: cAMP-response element binding protein. ESRR: estrogen-related receptor alpha. E2F: E2F transcription factor 7. MYT1: myelin transcription factor 1. EGRF: early growth response factor. NRSE: neuron-restrictive silencer factor. ETSF: ETS1 factor. LHXF: LIM homeodomain transcription factor. PARF: PAR-domain basic leucine zipper transcription factor. NF1: nuclear factor 1. TATA: TATA box. SF1: vertebrate steroidogenic factor 1. NEUR: neuroD. FOX: forkhead domain factor. HEAT: heat shock factor.

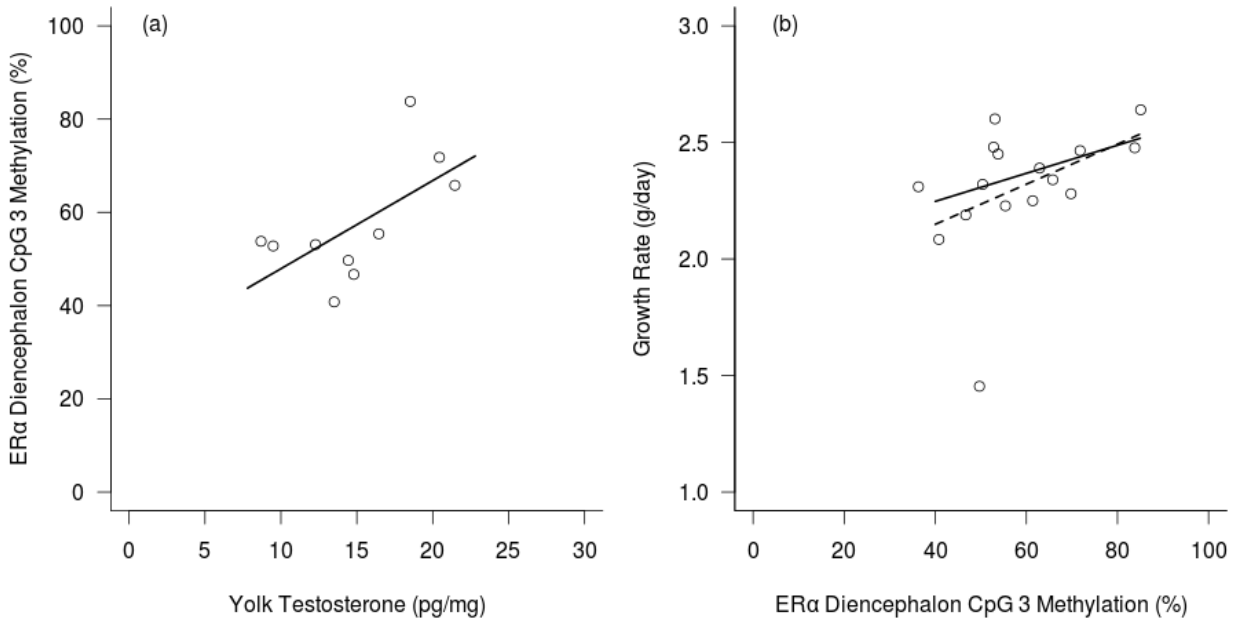

Figure 4.3. Relationships between (a) yolk testosterone concentration and percent DNA methylation of CpG site 3 in the putative promoter region of estrogen receptor alpha (ERα) in the diencephalon (solid line is linear regression line) and (b) percent DNA methylation of ERα CpG site 3 in the diencephalon and nestling growth rate (solid line is robust regression line and dashed line is linear regression line).

CHAPTER 5

MOLECULAR MECHANISMS MEDIATING THE EFFECTS OF PRENATAL TESTOSTERONE ON ADULT AGGRESSIVE PHENOTYPES.[A]

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ABSTRACT

Maternal hormones are transferred to offspring during prenatal development in response to heterogeneity in the maternal environment. Many researchers have postulated that prenatal maternal hormones can generate adaptive phenotypic plasticity. For example, females in more competitive environments tend to allocate more testosterone to their developing offspring, and these offspring exhibit more aggressive behaviors throughout life, thus, matching offspring behavioral phenotype with the prevailing social environment. Despite the importance of this maternal effect in shaping aggressive behaviors, no study has determined the proximate mechanisms facilitating these long-term changes. Here, we explored the molecular mechanisms mediating changes in aggressive behavior in male and female songbirds using egg injections of testosterone or the vehicle and examined genome-wide changes in neural gene expression. Both sexes displayed more aggressive behaviors if they came from testosterone-injected eggs and hundreds of genes were differentially expressed, particularly in the male hypothalamus. Many of the genes were associated with "non-traditional" moderators of aggression (e.g., genes in the nitric oxide and serotonin pathways) and were directly related to aggressive phenotype in males. These results provide evidence for mechanisms facilitating the long-term effects of prenatal hormone exposure on behavioral plasticity.

Keywords: yolk testosterone, avian, gene expression, aggression, zebra finch

INTRODUCTION

There is growing evidence that a female's phenotypic response to her environment can generate non-genetic, phenotypic plasticity in her offspring (i.e., maternal effect, Mousseau and Fox 1998; von Engelhardt and Groothuis 2011). Maternal effects can adaptively program offspring for the prevailing environment (Pilz et al. 2004; Storm and Lima 2010; Meylan and Clobert 2005; Dantzer et al. 2013) or create maladaptive changes if the maternal environment is not representative of the offspring's postnatal experience (Gluckman and Hanson 2004). One way females communicate the current environment to their offspring is by transferring maternal hormones (von Engelhardt and Groothuis 2011). For example, females experiencing more competitive, aggressive environments generally transfer greater concentrations of testosterone to developing offspring (Schwabl 1997; Mazuc et al. 2003; Dantzer et al. 2011; Bentz et al. 2013; but see Bentz et al. 2016a). Offspring exposed to more prenatal testosterone exhibit more aggressive behaviors throughout life (Strasser and Schwabl 2004; Dloniak et al. 2006; Eising et al. 2006; Partecke and Schwabl 2008; Müller et al. 2009), which is beneficial if offspring also experience a competitive environment (Rosvall 2008).

Despite the adaptive potential in prenatal hormones shaping offspring behaviors, few studies have examined the proximate mechanisms. Thus far, the effects of prenatal testosterone on testosterone sensitivity have been explored (Pfannkuche et al. 2011) and, while the hypothalamic-pituitary-gonadal (HPG) axis is well-studied in relation to aggressive behaviors (Soma 2006), moderators of aggression have been found across many pathways (Nelson and Trainor 2007). For example, variation in aggression has been attributed to gene regulation in the brain associated not only with the HPG axis, but also the hypothalamic-pituitary-adrenal (HPA) axis, serotonin, nitric oxide, dopamine, somatostatin, histamine, and hypothalamic-

neurohypophysial-system (HNS; Nelson and Chiavegatto 2001; Nelson and Trainor 2007; Mukai et al. 2009; Filby et al. 2010). Therefore, long-lasting variation in aggressive behavior could be a result of prenatal testosterone acting on multiple neural pathways. Indeed, there is evidence suggesting maternal environments can influence genome-wide gene expression in embryoes (e.g., maternal predation risk, Mommer and Bell 2014).

Here, we explore the molecular mechanisms mediating changes in gene expression in two socially sensitive brain regions, the nucleus taenia of the amygdala (TnA) and the hypothalamus (Goodson 2005), in both male and female zebra finches (*Taeniopygia guttata*) exposed to experimentally elevated prenatal testosterone. Zebra finches provide a good system to investigate the mechanisms mediating the effects of prenatal hormones, because their young develop externally in eggs, yolk hormones vary substantially (Schwabl 1993), and genomic information is available (Warren et al. 2010). We tested the hypothesis that experimentally increased prenatal testosterone would result in (1) long-term increased aggressive behaviors in both sexes and (2) these behavioral changes would be accompanied by genome-wide changes in gene expression, specifically genes in the behaviorally sensitive pathways mentioned previously. This study is the first to examine genome-wide changes in gene expression as a mechanistic mediator between prenatal hormones and long-lasting changes in social behavior.

METHODS

Animal subject details

Male and female normal grey mutation zebra finches (approx. 1 yr. old) were randomly assigned to breeding pairs and individually housed in standard cages (43 x 43 x 38 cm) with a light dark cycle of 14:10h with two perches, a nest-box, and burlap ribbon as nesting material.

They were provided with a mixed seed diet, water, and cuttlebone ad libitum, and checked and refreshed daily. Offspring resulting from the experimental manipulation were reared in the parental cage until they reached 50 days post-hatch and their sexually dimorphic adult plumage was visible, at which point they were placed in same-sex flocks of up to 4 birds in standard cages. All animals were treated in accordance with University of Georgia's Institutional Animal Care and Use Committee (AUP #A2014-03-014-Y2-A0).

Prenatal hormone manipulation

We bred 20 individually housed zebra finch pairs and injected all eggs laid in a clutch according to Winter et al. (2013) with testosterone (500 pg testosterone in 5 μl peanut oil) or the control vehicle (5 μl peanut oil) on the morning eggs were laid as previously used in this species (Sandell et al. 2007). The treatment assigned to the first egg in a clutch was randomized and subsequent eggs received alternating treatments. We ultimately obtained 24 female offspring (n = 15 control; n = 9 testosterone) and 24 male offspring (n = 8 control; n = 16 testosterone).

Measures of offspring phenotype

Once offspring reached sexual maturity (i.e., displayed sexually dimorphic plumage; > 60 days post-hatch), aggression scores were assigned in individual trials. Subjects were isolated in a cage for two days, after which a novel same-sex individual of similar mass (+/- 1.0 g) was placed in the subject's cage for 15 min between 0700-1200 hrs. The aggression score is the number of aggressive actions: bill fencing (jab with bill), displacement (driven off perch), and chasing (follow displaced bird; Adkins-Regan and Robinson 1993; Bolund et al. 2007; Ardia et al. 2010) performed by the subject toward the intruder. We performed two trials for each individual on

separate days to calculate consistency of aggressive behaviors and the aggression score is the average of these trials. Three male and three female offspring from testosterone- and control-injected eggs were decapitated 30 min after an aggression trial was initiated to obtain tissue from the TnA and the hypothalamus (see 'Tissue dissection and library preparation' below).

In addition to measuring aggression in adults, we also measured other traits potentially affected by prenatal testosterone exposure, including: mass and tarsus growth (e.g., Schwabl 1996a, Navara et al. 2006a), begging rate (e.g., Schwabl 1996a; von Engelhardt et al. 2006), the ratio of the 2nd to the 4th digit (2D:4D, McIntyre 2006), bill maturation (a testosterone-dependent secondary sex trait; Ardia et al. 2010; Tobler et al. 2013), and plasma testosterone (Pfannkuche et al. 2011; see Appendix C 'Supplemental Experimental Procedures' for details).

Tissue dissection and library preparation

We followed the recommended sample preparation as outlined by the Songbird Neurogenomics (SoNG) Initiative in Replogle et al. (2008). Briefly, brains were removed and placed in liquid nitrogen until they could be stored in a -80^{o}C freezer. On average, it took 33.8 s (+/- 1.9 s SE) from cage removal to decapitation and 191.1 s (+/- 13.7 s SE) from cage removal to the brain being placed in liquid nitrogen. Optimal cutting temperature compound was used to embed brains, 50µm sections were cut with a cryostat, and a disposable 1 mm biopsy punch was used to make vertical punches to obtain the TnA and the hypothalamus according to the songbird brain atlas (Nixdorf-Bergweiler and Bischof 2007).

On average 2.5 mg (+/- 0.1mg SE) of tissue was homogenized with a Mini-BeadBeater-16 (BioSpec Products) using 0.2 g of 1 mm beads for 10s in TRIzol/TRI Reagent to extract total RNA with a Direct-zol RNA MicroPrep kit (Zymo Research) with a DNase treatment step to

remove DNA contamination. The quality of extracted RNA was checked on an Agilent Bioanalyzer 2100 and 500 ng of total RNA was used to construct stranded mRNA-Seq libraries using a KAPA Stranded mRNA-Seq Kit (Kapa Biosystems). Concentration of libraries was checked with a Qubit 2.0 Fluorometer (Life Technologies) and size/quality was checked with a Fragment Analyzer (Advanced Analytical Technologies). Libraries were divided into two pools and sequenced on two lanes of an Illumina NextSeq SE75 High Output Flow Cell at the University of Georgia Genomics Facility.

RNA-seq mapping, differential gene expression, and gene ontology enrichment

RNA-seq data was mapped to the *T. guttata* genome (Warren et al. 2010) version 3.2.4, downloaded from Ensembl release 88 in March 2017 (Yates et al. 2016). Reads were first trimmed for adapters and quality using Cutadapt version 1.9.1 (Martin 2011) supplied with TruSeq adapter sequences. An additional round of trimming was conducted using PRINSEQ version 0.20.4 (Schmieder and Edwards 2011) to remove further adaptor contamination. Reads were assessed for quality using FastQC version 0.11.4 (Andrews 2010) before and after each round of trimming. Trimmed reads were then mapped using Tophat2 version 2.1.1 (Kim et al. 2013) supplied with the *T. guttata* version 3.2.4 annotations.

Differential gene expression and FPKM values (Fragments Per Kilobase of transcript per Million mapped reads) was determined using the Cufflinks suite version 2.2.1 (Trapnell et al. 2010; Roberts et al. 2011; Trapnell et al. 2013). Protein coding gene and transcript expression was first quantified using Cuffquant. Differential gene expression was then determined using Cuffdiff2, which performed all pair-wise comparisons, increasing the number of tests and affecting the multiple testing correction. As only the comparison of control and treatment

between samples of matching sex and tissue were of interest; results were imported into R, comparisons not of interest were removed, and q-values (adjusted for the false discovery rate, FDR) were readjusted using the Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) and a cut off of $q < 0.01$ was applied.

Gene Ontology (GO) terms (Ashburner et al. 2000) for *T. guttata* were downloaded from ARK-Genomics (http://www.ark-genomics.org/). Enrichment analysis was performed for each comparison in R with the topGO (Alexa et al. 2016) package using the parentCHILD algorithm (Grossmann et al. 2007). GO terms were considered significant with $p < 0.01$.

Statistical analyses

All statistical analyses were performed using R version 3.2.1. In all models we included natal nest ID as a random effect and accounted for sex and egg order in the clutch, unless otherwise specified. Statistical details can be found in the Results and in Appendix C 'Supplemental Experimental Procedures'. All means are followed by +/- SE.

RESULTS

Prenatal testosterone creates more competitive phenotypes

Aggression was consistent across the two behavioral trials with an intra-class correlation of 0.55 (0.35-0.74 CI; Lessells and Boag 1987). We performed a linear mixed-effects model using the average aggression score and accounting for sex and egg order in the clutch with natal cage ID (to avoid pseudoreplication between siblings) and adult cage ID (to account for behavioral variation due to social experience; Ruploh et al. 2012,2013) as random effects. We calculated parameter-specific significance with a Satterthwaite approximation. The model

revealed that offspring from testosterone-injected eggs were more aggressive than controls ($F_{1,13.1}$ = 9.48, p = 0.009) and males were more aggressive than females ($F_{1,26.8}$ = 5.55, p = 0.03). Egg order in the clutch was not significant (p = 0.53). Given that sex was significant, we re-ran the models but separated the sexes and found that testosterone egg injections resulted in significantly more aggressive males ($F_{1,12.49}$ = 7.50, p = 0.02) but not females ($F_{1,15.32}$ = 3.99, p = 0.06; Figure 5.1); egg order was not significant (both p > 0.31).

For the additional phenotypic traits measured, we found that nestlings from testosterone-injected eggs grew significantly faster than controls ($F_{6,275}$ = 2.70, p = 0.015; Figure S5.1A). Although, mass differences between treatment groups were no longer significant in adults (mass at 50 days post-hatch: $F_{1,26}$=0.30, p = 0.59). Nestlings from testosterone-injected eggs begged longer on average (7.4s +/- 0.7) than controls (6.1 +/- 0.5; $F_{1,22}$ = 4.19, p = 0.05; Figure S5.2A). Adult offspring from testosterone-injected eggs also displayed a significantly lower plasma testosterone concentration following a conspecific intrusion than controls ($F_{1,14}$ = 5.18, p = 0.04; Figure S5.3). Treatment did not affect tarsus growth ($F_{5,227}$ = 1.45, p = 0.21; Figure S5.1B), how quickly individuals developed adult bill coloration ($F_{1,25}$ = 1.00, p = 0.33; Figure S5.2B), or the left ($F_{1,23}$ = 0.20, p = 0.66; Figure S5.2C) or right 2D:4D ($F_{1,24}$ = 1.70, p = 0.21; Figure S5.2D). For statistical details see Appendix C ‘Supplemental Experimental Procedures’.

Neural gene expression is modified by prenatal testosterone

The male hypothalamus showed the greatest response to the egg injection treatment with n = 612 differentially expressed genes between offspring from testosterone- and control-injected eggs (Figure 5.2), with n = 306 genes upregulated and n = 306 genes downregulated (Figure S5.4). There were n = 109 genes differentially expressed between treatment groups in the male

TnA (Figure 5.2); n = 66 genes upregulated and n = 43 genes downregulated (Figure S5.5). There were n = 57 genes differentially expressed in the female hypothalamus (Figure 5.2); n = 26 genes upregulated and n = 31 genes downregulated (Figure S5.6). There were n = 229 genes differentially expressed between treatment groups in the female TnA (Figure 5.2); n = 33 genes upregulated and n = 196 genes downregulated (Figure S5.7). A Principal Component Analysis of all annotated genes (n = 8,422) showed that female tissues and the male TnA exhibited extensive overlap in treatment groups, only the male hypothalamus showed distinct groupings by egg injection treatment (Figure S5.8). Males and females did not appear to respond to the prenatal testosterone treatment similarly as only n = 21 genes were differentially expressed in both sexes in the TnA and n = 11 genes in the hypothalamus (Figure 5.2). The two brain regions also did not show extensive overlap in differentially expressed genes; the hypothalamus and TnA shared n = 54 genes in males and n = 16 genes in females (Figure 5.2).

Potential moderators of gene expression were assessed by examining genes involved in epigenetic modifications, such as DNA methyltransferases (DNMTs) and histone deacetylases (HDACs). However, these genes were not differentially expressed between treatemtents in either sex or brain region with the exception of protein arginine methyltransferase 8 (PRMT8), which had significantly greater expression in the hypothalamus of males from testosterone-injected eggs than controls (log2 fold change = 1.17, q = 0.005).

The biological process GO terms enriched in the male hypothamus revealed processes such as motor coordination, metabolism, cognition, immune function, and behavior were affected by the treatment (e.g., locomotion, regulation of primary metabolic process, single-organism behavior, cognition, cellular response to estradiol stimulus, and negative regulation of B cell differentiation), while in the TnA immune function was most strongly affected (i.e.,

negative regulation of viral processes). In females, motor coordination and metabolism were affected in the TnA (e.g., hormone metabolic process, growth, and muscle cell proliferation) and in the hypothalamus response to wounding was the most strongly affected. For a complete list of the biological processes over-represented by the differentially expressed genes see Table S5.1.

Neural gene expression of behaviorally sensitive genes are correlated with aggression

To test the hypothesis that behaviorally sensitive genes are influenced by prenatal testosterone we specifically examined genes in pathways associated with aggression (Nelson and Trainor 2007). In the male hypothalamus, gene pathways associated with the HPA axis (corticotrophin-releasing hormone binding protein, CRHBP; corticotrophin-releasing hormone receptor 2, CRHR2), monoamines (phenylethanolamine N-methyltransferase, PNMT; dopa decaroxylase, DDC; 5-hydroxytryptamine receptor 1D, HTR1D; 5-hydroxytryptamine receptor 2C, HTR2C), nitric oxide (nitric oxide synthase 1, NOS1), somatostatin (somatostatin receptor 5, SSTR5), and melanocortin (melanocortin receptor 4, MC4R) along with brain-derived neurotrophic factor (BDNF), early growth response 1 (EGR1), neuropeptide Y receptor 2 (NPY2R), and rap guanine nucleotide exchange factor 2 (RAPGEF2) were significantly differentially expressed between treatments (Figure 5.3; Table S5.2). Males from testosterone-injected eggs had greater expression than controls of all behavioral genes, except for DDC, PNMT, MC4R, and NOS1 which were significantly downregulated (Figure 5.3; Table S5.2). No behavioral genes were differentially expressed in the male TnA. In females, genes involved in monoamine synthesis (tyrosine hydroxylase, TH; tryptophan hydroxylase 2, TPH2) were significantly downregulated in the hypothalamus of offspring from testosterone-injected eggs,

and genes associated with the HPA axis (CRHBP) and NPY were differentially expressed in the TnA (Table S5.2).

We next tested for relationships between the behaviorally relevant genes that were significantly affected by prenatal testosterone treatment and average aggression score using linear regression models and adjusting p-values with a Benjamini-Hochberg procedure (Benjamini and Hochberg 1995). Of the 17 genes tested, only four genes in the male hypothalamus were significant; DDC, NOS1, MC4R, and PNMT were all significantly, negatively related to average aggression scores (Figure 5.4; TableS5.3).

DISCUSSION

Prenatal exposure to experimentally elevated testosterone led to offspring having more competitive phenotypes; they were more aggressive as adults, and gained mass more quickly and had a greater begging rate as nestlings. Furthermore, offspring from testosterone-injected eggs displayed lower plasma testosterone after a conspecific intrusion, in agreement with Pfannkuche et al. (2011). These findings are congruent with those from other studies investigating the functional significance of prenatal maternal hormones on offspring (reviewed in von Engelhardt and Groothuis 2011); yet, despite this extensive body of research, there is little known about the underlying proximate mechanisms (Groothuis and Schwabl 2008). Studies more broadly investigating the prenatal effects of maternal environment on offspring have found genome-wide changes in gene expression (Mommer and Bell 2014) and offer evidence that DNA methylation patterns may also be affected (Heijmans et al. 2008; Rubenstein et al. 2015; Bentz et al. 2016b). Here, we provide some of the first experimental evidence that prenatal hormones can generate

long-lasting variation in aggressive behaviors by changing expression patterns of numerous neural genes in a sex-specific manner.

Sex-specific effects of prenatal testosterone

We found that the effect of yolk testosterone on aggressive behaviors was more pronounced in males than females and, accordingly, males also had a greater number of differentially expressed genes between treatment groups. Several studies have found evidence that males are more sensitive to prenatal testosterone than females (Tobler and Sandell 2007; von Engelhardt et al. 2006; Ruuskanen and Laaksonen 2010; Pfannkuche et al. 2011; Riedstra et al. 2013; but see Schwabl 1993; Strasser and Schwabl 2004; Eising et al. 2006; Partecke and Schwabl 2008). The sex-specific effects of yolk testosterone are often attributed to differences in androgen sensitivity during development (i.e., timing or location of androgen receptors or concentration of enzymes used for testosterone metabolim; Sockman et al. 2008). However, timing and location of gene expression associated with testosterone sensitivity and metabolism is not sexually dimorphic in embryonic zebra finches (Perlman et al. 2003; Perlman and Arnold 2003). Thus, an alternative explanation is that males may have higher endogenous androgen production during development (Woods et al. 1975; Tanabe et al. 1983) and, when combined with the exogenous addition of testosterone injections, causes more pronounced effects (Ruuskanen and Laaksonen 2010). Zebra finches do go through sex-specific endocrine changes in the first weeks after hatching (Adkins-Regan et al. 1990; Vockel et al. 1990) that may still be shaping gene expression patterns.

Males and females also differed in which brain regions were most sensitive to the testosterone treatment; the hypothalamus in males and the TnA in females showed the greatest

differential expression between treatments. This difference could be explained by sex-specific activation of neural pathways in response to aggression in finches (Goodson et al. 2004). Females have greater immediate early gene responses to same-sex conspecifics in the TnA than males (Goodson et al. 2005). Another explanation, which is not necessarily mutually exclusive, is that differences could arise because we allowed birds to interact during aggression trials, such that results reflect both social arousal and motor system activation. Males did perform more aggressive actions than females during trials, and the hypothalamus is thought to play a larger role in behavioral performance while the TnA is involved with emotional processing (Mogenson et al. 1980; Cheng et al. 1999; Cardinal et al. 2002; Phelps and LeDoux 2005; Sagaspe et al. 2011). Regardless, the proximate reasons for the sex-specific effects of yolk testosterone are not clear and more studies should include both sexes.

HPA axis

The HPA axis regulates stress responses and several genes on this axis were differentially expressed between treatments. In the present study, both males and females from testosterone-injected eggs upregulated CRHBP, and CRHR2 was upregulated in males. CRHBP is responsible for binding corticotrophin-releasing factor and limiting the HPA axis (Laryea et al. 2012), and increased expression of CRHBP has been associated with increased aggression in mammals (Gammie et al. 2008). Similarly, CRHR2 promotes the reduction of anxiety, more prominently in males than females (Kishimoto et al. 2000; Coste et al. 2006). Evidence suggests genes in the HPA axis are sensitive to androgens; for example, androgens increase CRHR2 expression in the male hypothalamus (Weiser et al. 2008), suggesting these genes could be a target for regulation by androgens during prenatal development. Altogether, our data suggest that

prenatal testosterone increases expression of genes involved in downregulating the stress response, potentially leading to low baseline HPA axis functioning and more aggressive behaviors (Haller and Kruk 2003; Kruk et al. 2004; Summers et al. 2005).

Monoamines

Serotonin functions to inhibit impulsive behaviors and low levels of serotonin are associated with increased aggression (Sperry et al. 2003; Olivier 2004; Montoya et al. 2012). In the present study, both males and females downregulated enzymes responsible for the synthesis of serotonin; DDC (catalyzes the final step in serotonin biosynthesis) in males and TPH2 (the rate-limiting enzyme that catalyzes the first step of serotonin biosynthesis) in females. Furthermore, we found a negative significant relationship between DDC and aggression in males. Additionally, two serotonin receptors associated with aggression (HTR1D and HTR2C; Våge et al. 2010) were upregulated in the male hypothalamus. HTR1D is an autoreceptor that decreases serotonin release (Hoyer et al. 2002) and HTR2C is associated with an increased anxiety profile (Kimura et al. 2009; Li et al. 2012). Testosterone may have an inhibitory effect on the serotonin pathway (Birger et al. 2003; Sotomayor-Zárate et al. 2011) and DDC has been shown to interact with androgen receptors (Wafa et al. 2003), suggesting there is a possibility that prenatal testosreone could exert an organizational inhibitory effect on serotonin synthesis to increase aggression. Hu et al. (2015) recently showed that prenatal testosterone can increase HTR2C expression and anxiety in mammals.

Catecholamines (i.e., dopamine, norepinephrine, and epinephrine) are often positively associated with aggressive behaviors (McIntyre and Chew 1983; Korte et al. 1997; Volavka et al. 2004; Sørensen et al. 2005; Cheng and Muir 2007). Thus, it was unexpected when we found that

enzymes involved in catecholamine synthesis were downregulated in males and females from testosterone-injected eggs, including TH (the rate-limiting enzyme catalyzing the first step of catecholamine biosynthesis) in females, and DDC (catalyzes the synthesis of dopamine) and PNMT (catalyzes the synthesis of epinephrine) in males. In males, expression of DDC and PNMT were also negatively correlated with aggressive behaviors. The interaction between catecholamines and aggression may not be as clear as previously thought. The role of catecholamines appears to be dependent on the context and motive for the aggressive behavior (Bell and Hepper 1978). Indeed, some studies have found a negative effect of catecholamines on aggression (Höglund et al. 2005; Patki et al. 2015). Furthermore, catecholamines may act indirectly on behaviors through other neurological pathways (Hodge and Butcher 1975; Bell and Hepper 1987), thereby decreasing the clarity of the relationship with aggression. For example, androgens have been shown to both activate (Jeong et al. 2006) and suppress (Johnson et al. 2010) catecholamine synthesis. Thus, it is currently unclear what role the changes in catecholamine synthesis we found play and requires further investigation through direct experimental manipulation.

Nitric oxide

Nitric oxide is a signaling molecule synthesized by NOS1 that performs several biological functions involved with the circulatory system, immunity, and behaviors (Nelson 2005). There is strong support for a negative relationship between nitric oxide and aggressive behaviors (Demas et al. 1997; Kriegsfeld et al. 1997; Chiavegatto and Nelson 2003), but this effect seems to be restricted to males (Nelson et al. 1995; Gammie et al. 2000; but see Gammie and Nelson 1999). Thus, it was not surprising that we found NOS1 to be downregulated in males

from testosterone-injected eggs and for NOS1to be negatively associated with aggression. How the effects of nitric oxide on aggression are mediated are unclear, though there is some evidence suggesting it acts through serotonin or the HPA axis (Chiavegatto and Nelson 2003; Nelson 2005). Nevertheless, androgens have been shown to inhibit NOS (Kriegsfeld et al. 1997; Singh et al. 2000), which supports the idea that prenatal testosterone may have an organizational inhibitory effect on NOS1 to increase aggression.

Melanocortin

The melanocortin receptor MC4R increases energy expenditure and reduces food intake (Saper and Lowell 2014), and knockout studies show that deficiency results in increased growth (Huszar et al. 1997). In the present study, we found that males from testosterone-injected eggs had lower expression of MC4R, which was negatively related to aggression. Thus far, only MC5R has been associated with aggression via pheromones (Morgan et al. 2004; Ducrest et al. 2008). One possibility is that decreased MC4R could cause increased food intake (birds from testosterone-injected eggs did grow more quickly), which could result in increased food motivation and territoriality, leading to higher aggression. However, contrary to this idea is the finding that NPY (a stimulant of feeding behavior; Clark et al. 1984) was downregulated in individuals from testosterone-injected eggs in both males (increased NPY2R, an autoreceptor for NPY) and females (decreased NPY; Heilig 2004). Decreased NPY has been associated with increased aggression, potentially through an interaction with serotonin (Karl et al. 2004). Thus, our findings could reflect interactions between food motivation or growth and other pathways that influence aggression.

HPG axis

In the past, researchers studying the effects of yolk tesostorone had focused on the HPG axis (Soma 2006). Indeed, one of the few studies testing mechanisms of yolk testosterone found that it led to lower plasma testosterone production (Pfannkuche et al. 2011), which we also found. However, we did not find any key genes in the HPG axis that were differentially expressed between treatments. Estrogen receptors, aromatase, gonadotropin-releasing hormone, and the predicted androgen receptor (GenBank accession number: NC_011468.1) were present at detectable levels, but did not differ between treatments in males or females. One possibility is that this is an artifact of domestication; domestic zebra finches generally have higher levels of circulating steroids than wild zebra finches (Prior et al. 2017) which may cause the HPG axis to function differently. Alternatively, production or sensitivity to steroids in the gonads could have been affected, but this was not measured.

Mechanisms of differential gene expression

Given the large number of differentially expressed genes elicited by prenatal testosterone exposure, the next step is to consider how gene expression was altered. One hypothesis is that epigenetic modifications (stable changes in gene expression without altering DNA sequence; Holliday 1994) are how prenatal hormones exert their effects on gene expression. Targeted studies of DNA methylation in wild birds suggest that DNA methylation can function to translate the maternal environment to offspring (Rubenstein et al. 2015; Bentz et al. 2016b). Studies examining DNA methylation in response to prenatal environment have also found sex-specific results with stronger effects being observed in males (Tobi et al. 2009; Rubenstein et al. 2015), which would mirror our findings. A potential mechanistic link between prenatal testosterone and

methylation is that metabolites of testosterone (i.e., estrogens) can downregulate DNMTs (Yamagata et al. 2009), but it is unclear if this also occurs in avian species. We did not find that DNMTs or HDACs were downregulated, but we did find that PRMT8 (a protein arginine methyltransferase; Lee et al. 2005) had significantly greater expression in males from testosterone-injected eggs. PRMTs can regulate transcription factors and histones through methylation and is a direct way in which gene expression can be epigeneticlally regulated (Bedford and Clarke 2009).

Considerations for future studies

In the current study, we were unable to separate whether the behavioral effect we saw in adults was a result of the transient early activational or permanent organizational effects of prenatal testosterone. It is possible that the long-term effects of prenatal hormones are indirect rather than organizational. Early activational effects of testosterone may shape the early competitive environment that then dictates behaviors as adults (Carere and Balthazart 2007). The social environment experienced early in life can have long-term effects on social behavior (Groothuis and Meeuwissen 1992; Ruploh et al. 2012, 2013) and gene expression (Banerjee et al. 2012). Furthermore, prenatal hormones could have organizational effects on a subset of genes that then create a cascade of indirect changes through hormonal autoregulatory responses, a possibility proposed by Pfannkuche et al. (2011). We do account for both rearing and adult environment as random effects in our models to help statistically control for these factors; nevertheless, future studies should examine the effects of the prenatal environment across different life stages within a study.

Conclusions

Maternal effects can act as mechanisms for adaptive phenotypic response to environmental heterogeneity, providing a way for females to help their offspring cope with the current environment. However, without knowing the mechanisms that mediate maternal effects, we cannot begin to fully understand how they fit into larger ecological and evolutionary frameworks. Phenotypic plasticity resulting from prenatal hormone exposure seems to be, in part, moderated by changes in neural gene expression in a sex-specific manner. This helps shed light on the ecological and evolutionary significance of molecular mechanisms in mediating transgenerational adaptive plasticity.

FIGURES

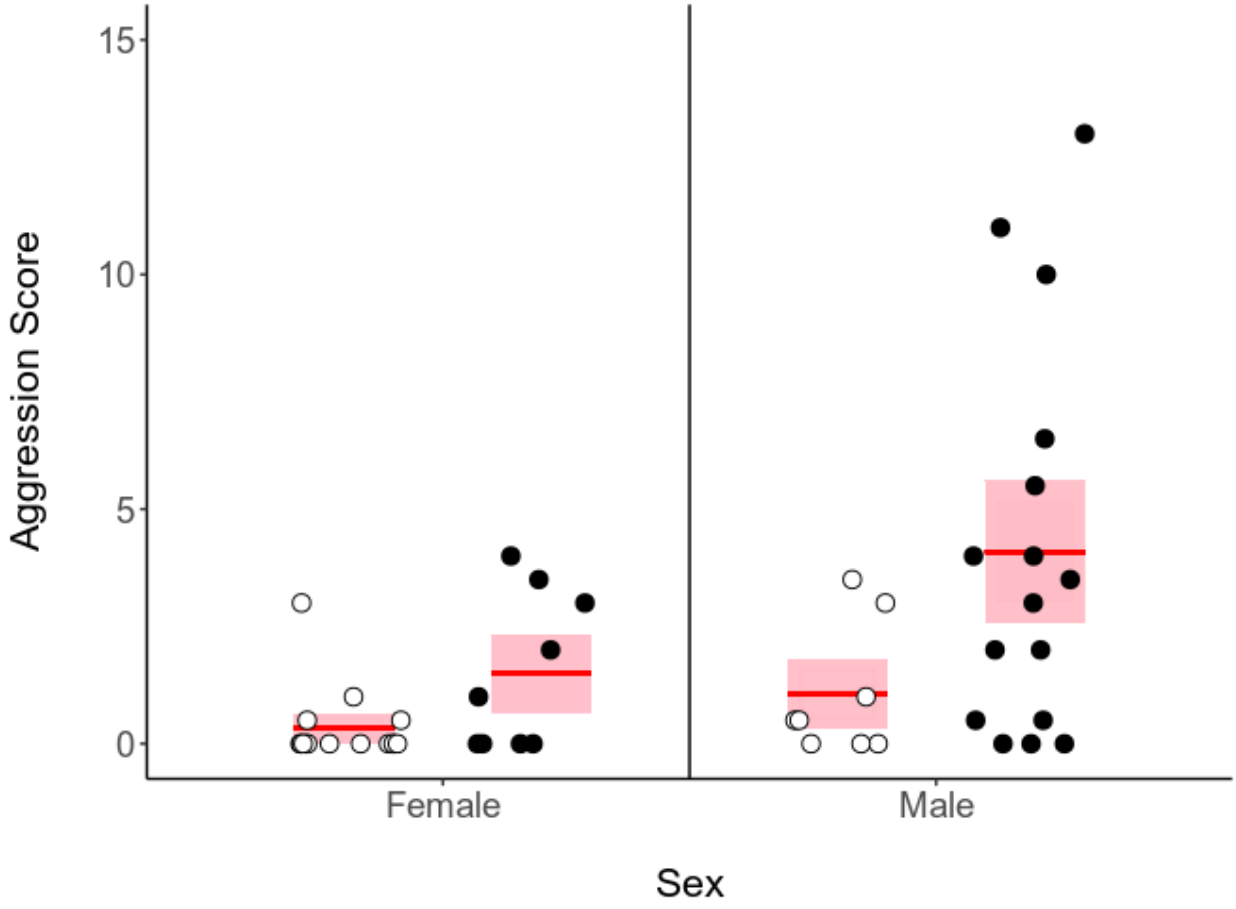

Figure 5.1. Relationship between average aggression score and treatment (control, open circles, or testosterone, filled circles, egg injection) within each sex. The red line represents the mean and the shaded area is the SE.

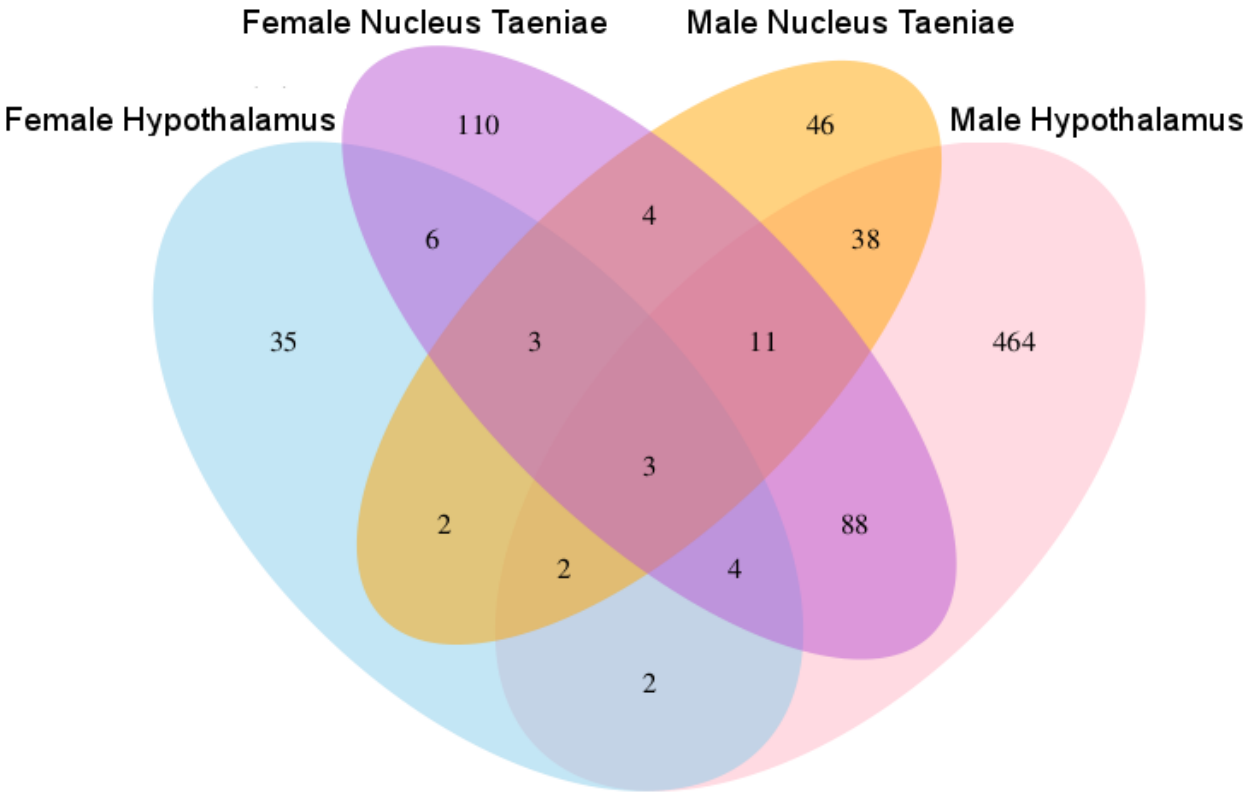

Figure 5.2. Venn diagram of differentially expressed genes between control and testosterone egg injection treatments for males and females and in hypothalamus and nucleus taeniae brain regions. Gene Ontology analysis was performed to identify which biological functions are represented by these differentially expression genes (see Table S5.1).

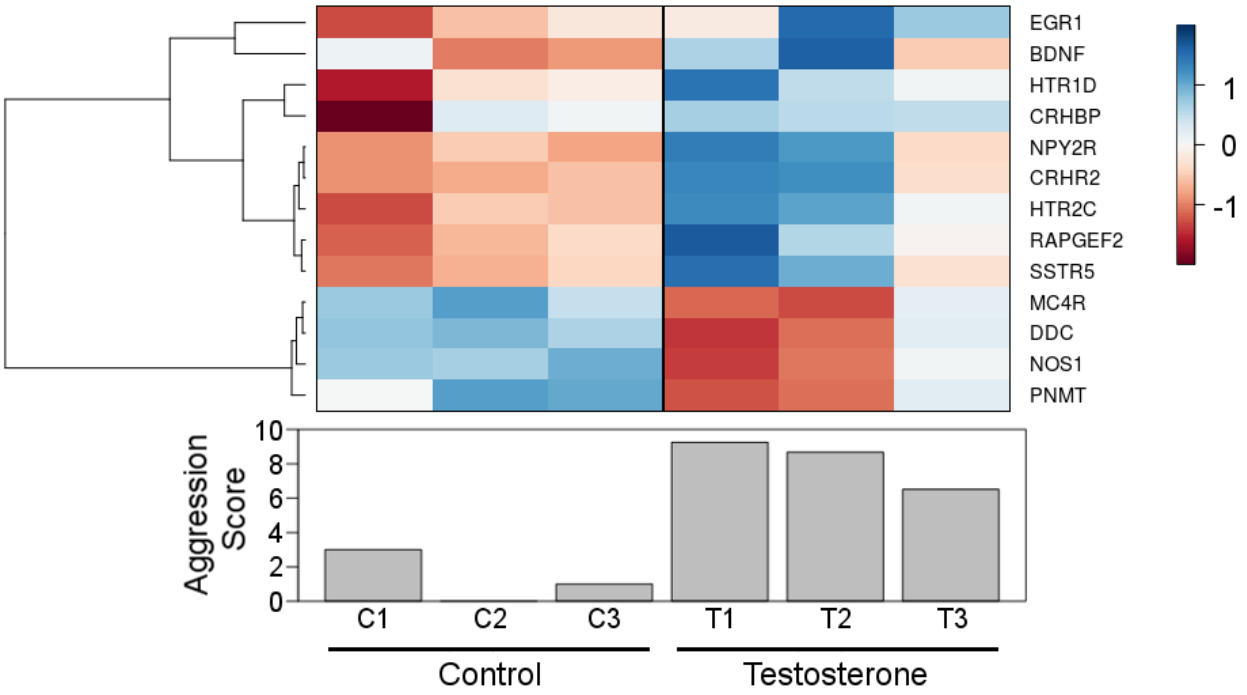

Figure 5.3. A subset of genes in the male hypothalamus associated with aggressive behaviors (for all genes see Figure S5.4) for individuals from control and testosterone-injected eggs and their average aggression scores. Rows represent genes and columns represent individuals from control (n = 3) or testosterone-injected (n = 3) eggs with their corresponding average aggression score. Blue = upregulated and red = downregulated compared to the mean value of a gene across all samples. Log(1+FPKM) data are scaled (Z-score) so that units of change are standard deviations from the mean. Genes were clustered using Pearson correlation to construct the dendrogram. Abbreviations: EGR1, early growth response protein 1; BDNF, brain-derived neurotrophic factor; HTR, 5-hydroxytryptamine (serotonin) receptors; CRHBP, corticotrophin-releasing hormone binding protein; NPY2R, neuropeptide Y receptor Y2; CRHR2, corticotropin releasing hormone receptor 2; RAPGEF2, rap guanine nucleotide exchange factor 2; SSTR5, somatostatin receptor 5; MC4R, melanocortin 4 receptor; DDC, dopa decarboxylase; NOS1, nitric oxide synthase 1; PNMT, phenylethanolamine N-methyltransferase.

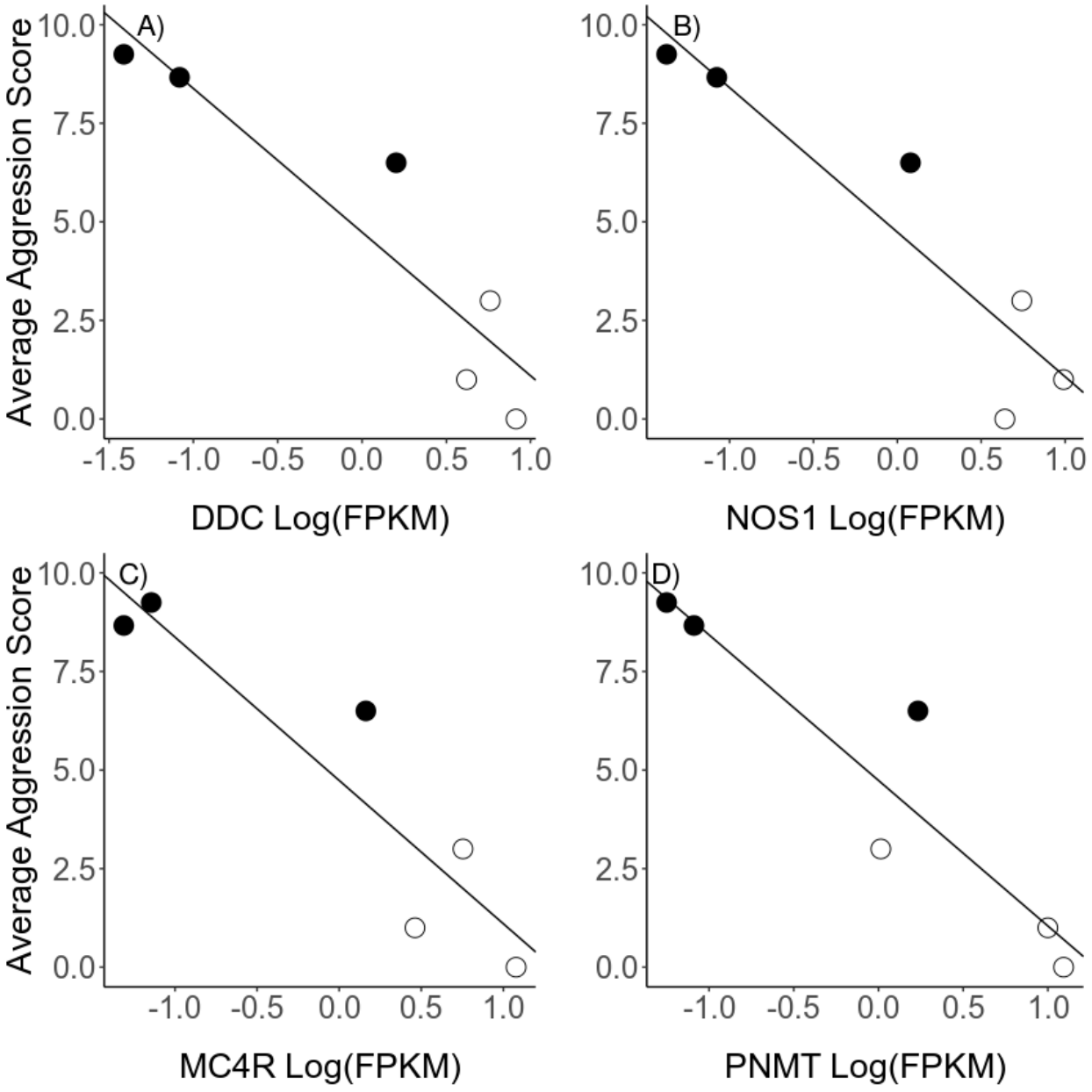

Figure 5.4. Relationship between average aggression score and Z-score of the Fragments Per Kilobase of transcript per Million mapped reads (Log_{10}(1+FPKM)) for A) dopa decarobxylase (DDC), B) nitric oxide synthase 1 (NOS1), C) melanocortin receptor 4 (MC4R), and D) phenylethanolamine N-methyltransferase (PNMT) in the male hypothalamus. Filled circles are males from testosteorne-injected eggs and open circles are from control eggs.

CHAPTER 6

CONCLUSIONS

Previous studies have explored environmental causes of intraspecific variation in prenatal hormone allocation and the functional consequences for offspring (reviewed in von Engelhardt and Groothuis 2011), which have built the foundation for studies to now determine the mechanisms mediating these effects. My dissertation sought to identify why females respond to competitive environments with different yolk testosterone allocation strategies and also to determine the proximate mechanisms linking maternal hormones to long-term changes in offspring behavior. I found that colonial species do not allocate more yolk testosterone in response to competitive environments, unlike solitary species (Chapters 2 and 3). This work shows that ultimate explanations of maternal effects should be contextualized with life-history traits. I then showed that natural variation in yolk testosterone allocation is correlated with changes in DNA methylation of a neural gene (estrogen receptor alpha) and growth in juvenile offspring (Chapter 4). Next, experimental manipulation of yolk testosterone led to changes in neural gene expression of hundreds of genes, several of which are related to behaviors (e.g., serotonin and nitric oxide pathways; Chapter 5). These data suggest that the effects of prenatal testosterone act by changing gene expression, possibly via epigenetic mechanisms.

The work in this dissertation raises several questions and concerns for future work. The analyses in Chapters 2 and 3 demonstrate that future studies need to diversify the number of species studied to create a more comprehensive picture of why specific maternal hormone

allocation strategies have evolved. Maternal hormone allocation response to competitive environments has only been studied in 17 species across two decades and these species are biased toward altricial passerines breeding in temperate latidutes (von Engelhard and Groothuis 2011). This makes it difficult to examine ultimate explanations of this maternal effect in the context of developmental strategies (altricial vs precocial), latitude (tropical vs temperate), and several other life history traits (e.g., trophic level, breeding seasonality, etc.). Additionally, future studies could look for indications of divergent evolution in populations of species that have different life-history strategies; for example, some species can nest in both territorial and colonial patterns (e.g., red-necked grebes, *Podiceps grisegena*; Klatt et al. 2004).

Additionally, the work in Chapters 2 and 3 brings forth several questions regarding the mechanisms of maternal hormone allocation. The assumption that all females should respond to increasing competitive aggression by increasing yolk testosterone allocation comes from the assumption that female plasma and yolk hormone responses to aggression are linked and positively correlated (Schwabl 1996b; Jawor et al. 2007). However, the relationship between plasma and yolk testosterone in response to aggression is not clear with some studies finding negative relationships (e.g., Navara et al. 2006b). Thus, the work presented in Chapters 2 and 3 supports the idea that the mechanisms regulating yolk and plasma testosterone are independent (Hackl et al. 2003; Okuliarova et al. 2011; Egbert et al. 2013). There is evidence that natural selection can shape yolk and plasma hormone allocation separately (Okuliarova et al. 2011), which could explain the differences in yolk allocation strategy in colonial and solitary species (Chapter 2), and how a female can respond aggressively but not increase yolk testosterone allocation (Chapter 3). Future research will need to identify the mechanisms allowing steroid transfer to the yolk and hormonal plasma responses to be independent, exploring areas such as

steroidogenic enzymes in follicles (Payne and Hales 2004; Egbert et al. 2013), neural steroid responses to aggression (Soma et al. 2008; Schlinger and Remage-Healey 2012), metabolic conjugation (Paitz and Bowden 2008), and enzymatic barriers or membrane transporters (Moore and Johnston 2008). Furthermore, if yolk and plasma testosterone are moderated independently, then ultimate questions about points of selection also need to be addressed (e.g., is selection acting on mothers, on juvenile offspring, or on adult offspring). Few studies have explicitly tested the fitness consequences (survival or reproductive success) of yolk testosterone (Pilz et al. 2004; Muriel et al. 2015).

The data presented in Chapter 4 shed light on the potential mechanisms that mediate the effects of environmentally elicited yolk testosterone on offspring phenotype, and suggest avenues for future research. For example, future studies could administer steroid receptor antagonists or aromatase inhibitors into yolk to directly test the hypothesis that yolk testosterone acts through estrogen receptor alpha. Further analysis of the structure of the estrogen receptor alpha promoter in passerines would also help to provide insights into the mechanisms that regulate its expression and the relevance of these mechanisms to maternal effects.

To fully determine if the effects of prenatal hormones seen in juvenile (Chapter 4) or adult (Chapter 5) stages are a result of transient early activational or permanent organizational effects the work in this dissertation should be repeated at several different life stages within a study. Yolk testosterone could shape the early competitive environment through activational effects that then dictates behaviors as adults (Carere and Balthazart 2007) or may create organizational changes in gene expression that then create a cascade of indirect changes through hormonal autoregulation (Pfannkuche et al. 2011). Postnatal experiences have also been shown to influence DNA methylation, gene expression, and behaviors (Weaver et al. 2004; Champagne

et al. 2006; Murgatroyd et al. 2009). Thus, when examining the effects of prenatal testosterone, individuals should be tested prior to and immediately after birth/hatch, as juveniles, and as adults.

Altogther, this dissertation examines previously unexplained variation in maternal effects and the mechanisms that mediate them. This work includes the first use of a comparative approach to examine a maternal effect using maternal responses (i.e., effect sizes) rather than species average values (Chapter 2). This work is also one of the first to examine DNA methylation (Chapter 4) and genome-wide changes in gene expression as a mechanistic mediator between prenatal hormones and long-lasting changes in social behavior (Chapter 5). Ultimately, this work helps elucidate the adaptive role of maternal effects and helps us predict how species will respond to changes in their social environment. Furthermore, this work clarifies what molecular mechanisms mediate the effects of maternal testosterone on offspring, helping us better understand the environmental and molecular causes of phenotypic plasticity.

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APPENDIX A

CHAPTER 2: SUPPLEMENTAL INFORMATION[A]

[A]Bentz, A.B., Becker, D.J. and Navara, K.J. 2016. Evolutionary implications of interspecific variation in a maternal effect: a meta-analysis of yolk testosterone response to competition. R. Soc. Open Sci. 3: 160499. Reprinted here with permission of publisher.

ADDITIONAL TABLES AND FIGURES

Table S2.1. Details of species included in the meta-analysis, including the Fisher's *Z* transformed effect size and both study- and species-specific moderators. Moderators are scored as follows: experiment type, 0 = no manipulation, 1 = indirect manipulation, and 2 = direct, experimental manipulation; coloniality, 1 = solitary, 2 = semi-colonial (1-10 pairs), and 3 = colonial (>10 pairs); nest type, 1 = closed and 2 = open; development type, 1 = altricial and 2 = precocial; and mating type, 1 = monogamous and 2 = polygamous and cooperative breeders. Percent of extra-pair copulations (% EPC) is a weighted average across studies. References for life-history traits follow each species' scientific name. References for specific studies follow corresponding effect size, sample size, and experiment type (if multiple studies within a species used different experiment types). References used to calculate % EPC follow % EPC values.

Scientific name	Effect size (Fisher's Z)	*n*	Experiment type	Coloniality	Nest type	Days to fledge	Clutch size	Dev. type	Mating type	% EPC
Ficedula albicollis[1]	0.53[2]	23[2]	2	1	1	16	6	1	2	39.5[3,4]
Fulica americana[5]	1.07[6]	24[6]	0	1	2	1	7.5	2	1	-
Crotophaga ani[7]	0.16[8]	48[8]	0	2	2	10	5	1	2	-
Tachycineta bicolor[9]	0.71[10], 0.93[10], 0.63[11]	18[10], 10[10], 16[11]	1[10], 0[10,11]	1	1	20	5.5	1	1	69.2[12–14]
Athene cunicularia[15]	0.14[16]	47[16]	0	2	1	48.5	8	1	1	-
Passer domesticus[17]	1.00 [18], 0.85[19]	10[18], 23[19]	1[18],2[19]	2	1	14	4.5	1	2	28.7[20,21]
Larus fuscus[22]	-0.06[23]	22[23]	1	3	2	2	3	2	1	-
Guira guira[24]	0.07[25]	35[25]	0	2	2	5.5	6	1	2	-
Parus major[26]	0.13[27]	128[27]	0	1	1	19	8	1	1	29.5[28,29]
Chroicocephalus ridibundus[22]	-0.68[30], -0.76[30]	13[30], 16[30]	0	3	2	10	2.5	2	1	33.3[31]
Hirundo rustica[32]	-0.63[33]	23[33]	0	3	2	20	4.5	1	1	34.7[34]
Sialia sialis[35]	0.73[36], 0.52[37]	28[36], 19[37]	2[36],0[37]	1	1	18	4.5	1	1	25.9[38–40]
Philetairus socius[41]	0.15[42], 0.01[43], 0.05[44]	28[42], 27[43], 18[44]	0	3	2	15	4	1	2	-
Sturnus vulgaris[45]	0.30[46], 0.56[47]	57[46], 24[47]	1[46], 0[47]	2	1	21	4.5	1	2	30.6[48,49]
Taeniopygia guttata[50]	0.35[Bentz et al. unpubl]	16[Bentz et al. unpubl]	2	3	2	15	5	1	1	5.3[51,52]
Coturnix japonica[53]	0.69[54]	20[54]	2	1	2	19	6.5	2	2	-
Sialia mexicana[55]	0.65[56]	20[56]	1	1	1	21	4.5	1	1	45.6[57–59]

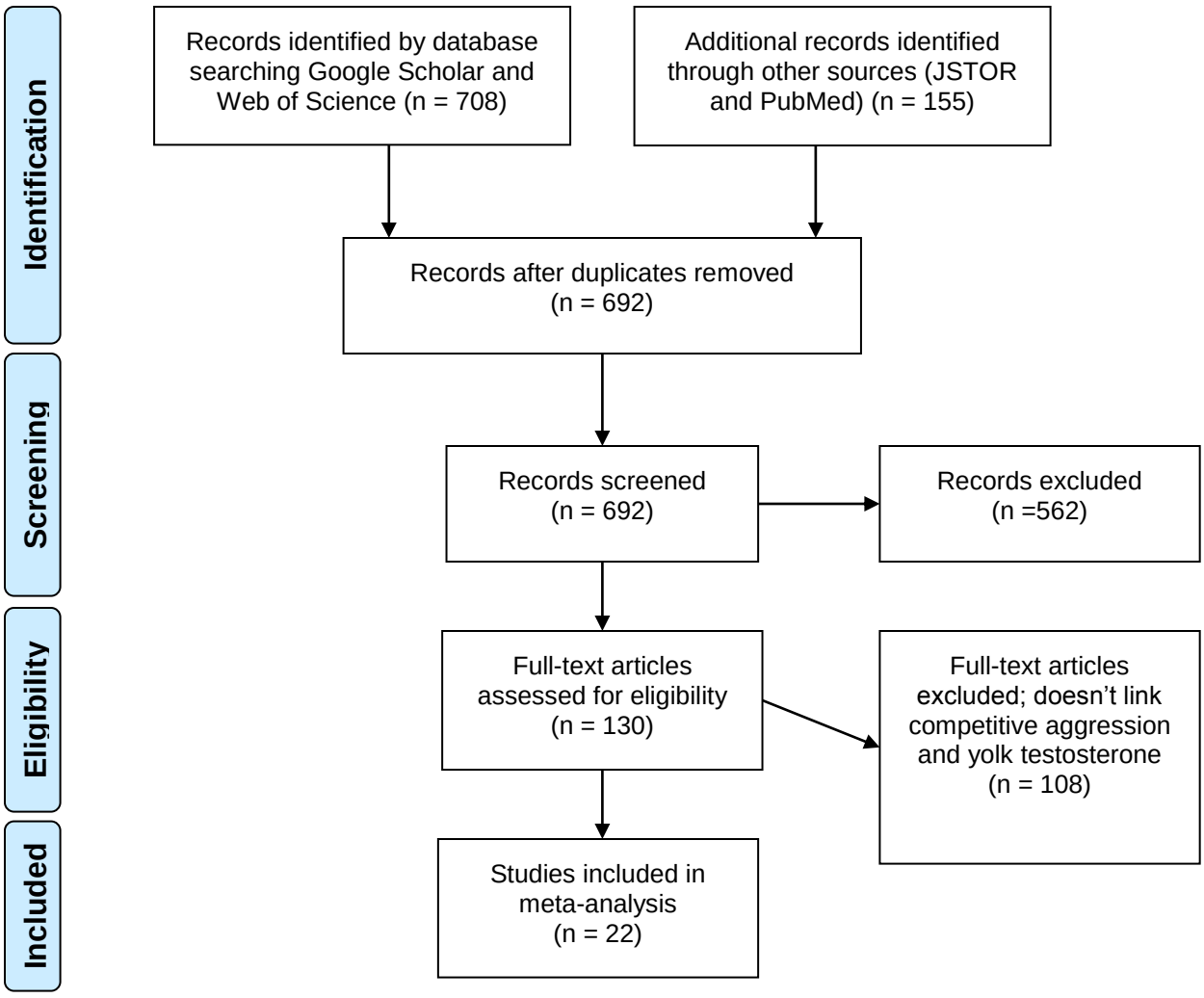

Figure S2.1. Flow diagram for documenting the data collection and inclusion process according to PRISMA style ([60]; see "Methods" in CHAPTER 2 for detailed criteria for inclusion and exclusion of published articles within our analysis).

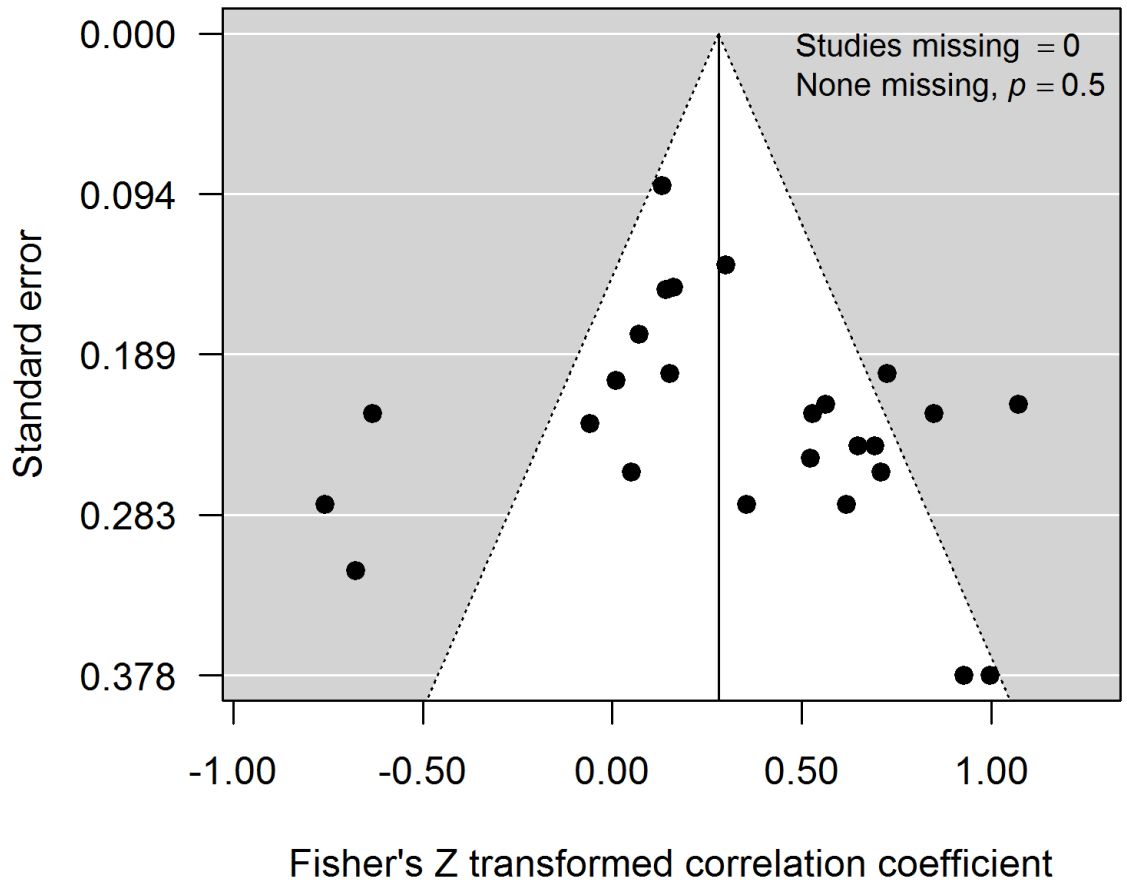

Figure S2.2. Funnel plot illustrating the relationship between Fisher's *Z* effect size and standard error, where each data point is an individual study on the relationship between competitive environment and yolk testosterone response.

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APPENDIX B

CHAPTER 4: SUPPLEMENTAL INFORMATION[A]

[A]Bentz, A.B., Sirman, A.E., Wada, H., Navara, K.J. and Hood, W.R. 2016. Relationship between maternal environment and DNA methylation patterns of estrogen receptor alpha in wild Eastern Bluebird (*Sialia sialis*) nestlings: a pilot study. Ecol. Evol. 6: 4741-4752. Reprinted here with permission of publisher.

ADDITIONAL TABLE

Table S4.1. All potential transcription factor binding sites (matrix similarity >72%) on the putative Eastern Bluebird (*Sialia sialis*) ERα promoter region according to MatInspector (Genomatix).

Matrix	Detailed Matrix Information	Start position	Matrix sim.	Sequence
V$BRIGHT.01	Bright, B cell regulator of IgH transcription	1	0.937	gaaaaATTAaaagatcagtaa
V$HBP1.01	HMG box-containing protein 1	15	0.895	tcagtaagAATGaagtaacattt
V$CREB1.02	cAMP-responsive element binding protein 1	19	0.936	taagaATGAagtaacatttag
V$VBP.01	PAR-type chicken vitellogenin promoter-binding protein	20	0.914	aagaatgaaGTAAcatt
V$SOX1.04	SRY (sex determining region Y)-box 1, dimeric binding sites	23	0.796	aatGAAGtaacatttagacggac
V$CEBPE_ATF4.01	Heterodimer of CEBP epsilon and ATF4	23	0.945	aatgaaGTAAcat
V$E4F.01	GLI-Krueppel-related transcription factor, regulator of adenovirus E4 promoter	24	0.893	atgAAGTaacatt
V$SMARCA3.01	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	30	0.981	taACATttaga
V$NF1.01	Nuclear factor 1	49	0.842	tctTTGGtataactccagaac
V$LHX3.02	LIM-homeodomain transcription factor LHX3	57	0.838	ataactccagaacTAATtgtttc
V$LHX4.01	LIM homeobox 4, Gsh4	61	0.866	ctccagaactAATTgtttctgaa
V$DLX3.01	Distal-less 3 homeodomain transcription factor	61	0.944	ctccagaacTAATtgtttc
V$NOBOX.01	Homeobox containing germ cell-specific transcription factor NOBOX	63	0.982	ccagaacTAATtgtttctg
V$GSH2.01	Homeodomain transcription factor Gsh-2	64	0.959	cagaacTAATtgtttctga
V$HOXA3.01	Homeobox A3	65	0.87	agaacTAATtgtttctgaa
V$NKX12.01	NK1 homeobox 2, Sax1-like	65	0.907	agaactAATTgtttctg

Matrix	Detailed Matrix Information	Start position	Matrix sim.	Sequence
V$PCE1.01	Photoreceptor conserved element 1	65	0.944	agaacTAA Ttgtttctg
V$S8.01	Binding site for S8 type homeodomains	65	0.995	agaacTAA Ttgtttctgaa gt
V$PAX7.01	Paired box 7 homeodomain-binding motif	68	0.837	actaattGT TTctga
V$GABPB1.01	GA repeat binding protein, beta 1)	72	0.8	attgtttCTG Aagtgatgtt
V$ATF2.01	Activating transcription factor 2	74	0.871	tgtttcTGA Agtgatgttt aa
V$NKX31.04	NK3 homeobox 1	76	0.953	tttctgaAG TGatgtttaa
O$MTATA.01	Muscle TATA box	87	0.854	atgttTAA Accaacgtc
V$ESRRA.05	Estrogen-related receptor alpha, homodimer DR5 binding site	97	0.74	caacgtcgg cacAAGG caagggc
V$PAX3.01	Pax-3 paired domain protein, expressed in embryogenesis, mutations correlate to Waardenburg Syndrome	101	0.772	gTCGGca caaggcaag ggc
V$SOX9.02	SRY (sex-determining region Y) box 9	102	0.948	tcggcACA Aggcaagg gctcctg
V$GKLF.01	Gut-enriched Krueppel-like factor	103	0.916	cggcacaag gcaAGGG c
V$SF1.01	SF1 steroidogenic factor 1	104	0.961	ggcaCAA Ggcaaggg
V$GC_SBE.01	GC-rich Smad1/5 binding element	114	0.953	aagggCTC Ctg
V$NF1.04	Nuclear factor 1	119	0.926	ctcctggcttg cagCCAG cac
V$MOK2.02	Ribonucleoprotein associated zinc finger protein MOK-2 (human)	126	0.983	cttgcagcca gcaCCTT gtaa
V$ZBTB3.01	Zinc finger and BTB domain containing 3	128	0.996	tgcaGCC Agca
V$NRSF.02	Neuron-restrictive silencer factor (11 bp spacer between half sites)	130	0.725	cagccaGC ACcttgtaat gcatattagtg ca
V$DBP.01	Albumin D-box binding protein	139	0.875	ccttgTAA Tgcatatta
V$POU3F3.01	POU class 3 homeobox 3 (POU3F3), OTF8	143	0.909	gtaatGCA Tattagt

Matrix	Detailed Matrix Information	Start position	Matrix sim.	Sequence
V$MEL1.02	MEL1 (MDS1/EVI1-like gene 1) DNA-binding domain 2	154	0.994	tagtgcaGATGaggagt
V$HOXB9.01	Abd-B-like homeodomain protein Hoxb-9	166	0.904	ggagttccTAAAagtta
V$BCL6.04	B-cell CLL/lymphoma 6, member B (BCL6B)	167	0.885	gagTTCCtaaaagttag
V$MYT1.02	MyT1 zinc finger transcription factor involved in primary neurogenesis	174	0.883	taaAAGTtagaga
V$GAGA.01	GAGA-Box	177	0.868	aagttAGAGagaggaagagggaggg
V$PRDM14.01	PR domain zinc finger protein 14	177	0.886	aagTTAGagagagga
V$GAGA.01	GAGA-Box	179	0.816	gttagAGAGaggaagagggagggaa
V$GAGA.01	GAGA-Box	181	0.891	tagagAGAGgaagaggagggaaga
V$ETV1.02	Ets variant 1	181	0.967	tagagagaGGAAgagggaggg
V$ZNF35.01	Human zinc finger protein ZNF35	185	0.961	gagaggAAGAggg
V$CKROX.01	Collagen krox protein (zinc finger protein 67 - zfp67)	191	0.919	aagaGGGAgggaagaagag
V$E2F7.02	E2F transcription factor 7	192	0.884	agagggaGGGAagaaga
V$NM23.01	NME/NM23 nucleoside diphosphate kinase1 and 2	192	0.908	agAGGGagggaagaag
V$MAZ.01	Myc associated zinc finger protein (MAZ)	193	0.914	gaggGAGGgaaga
V$SPZ1.01	Spermatogenic Zip 1 transcription factor	195	0.942	gGGAGggaaga
V$EVI1.07	Evi-1 zinc finger protein, carboxy-terminal zinc finger domain	197	0.905	gagggAAGAagagagag
V$FAST1.03	Forkhead box H1 (Foxh1)	211	0.914	gagaatgtgCATTttca
V$OCT3_4.02	POU domain, class 5, transcription factor 1	212	0.942	agaatgtGCATtttcagtg
V$SOX10.03	SRY (sex determining region Y)-box 10, dimeric binding sites	216	0.73	tgTGCAtttcagtgctcactct
V$PREB.01	Prolactin regulatory element-binding protein	220	0.901	catttTCAGtgctca

Matrix	Detailed Matrix Information	Start position	Matrix sim.	Sequence
V$MAFA.01	Lens-specific Maf/MafA-sites	226	0.948	cagtgctcac tcTGCAttt
O$ZSCAN4.01	Zinc finger and SCAN domain containing 4	229	0.837	tGCTCact ctgcatt
V$OCT3_4.02	POU domain, class 5, transcription factor 1	232	0.927	tcactctGC ATttgtttgt
V$ZNF263.02	Zinc finger protein 263, ZKSCAN12	251	0.92	atcctcCTC Cttgcc
V$GATA3.01	GATA-binding factor 3	264	0.914	ccgaGAT Tagagg
V$STAT.01	Signal transducers and activators of transcription	266	0.924	gagattaga GGAAtac
V$IK3.01	Ikaros 3, potential regulator of lymphocyte differentiation	270	0.873	ttagaGGA Atacc
V$AREB6.01	AREB6 (Atp1a1 regulatory element binding factor 6)	275	0.937	ggaatACC Tgtgt
V$PBX_HOXA9.01	PBX - HOXA9 binding site	289	0.792	gtgcTGCT ttattatga
V$OCT1.03	Octamer-binding transcription factor-1, POU class 2 homeobox 1 (POU2F1)	291	0.857	gctgctttAT TAtga
V$PBX_HOXA9.01	PBX - HOXA9 binding site	299	0.877	attaTGATt tctgctga
V$MAFF.01	Transcription factor MafF	307	0.83	ttctGCTG agcctcagaa taggt
V$FHXB.01	Fork head homologous X binds DNA with a dual sequence specificity (FHXA and FHXB)	315	0.842	agcctcAG AAtaggttc
V$HSF1.04	Heat shock factor 1	321	0.784	agaataggtt ctggtattTT TTtta
V$MTBF.01	Muscle-specific Mt binding site	332	0.97	tggtATTTt
V$TCFE2A.03	Transcription factor E2a (E12/E47)	350	0.995	gactgcCA GCtgccgat
V$OLIG2.01	Oligodendrocyte lineage transcription factor 2	352	0.994	ctgccaGC TGccg
V$PTF1.01	PTF1 binding sites are bipartite with an E-box and a TC-box (RBP-J/L) spaced one helical turn apart	354	0.779	gccaGCT Gccgatctc actgt
V$TBX20.02	T-box transcription factor TBX20	360	0.964	tgccgatctc actgttGAC Agtgaaagc
V$IRF2.01	Interferon regulatory factor 2	368	0.904	tcactgttgac agtGAAA gcagata
V$PRDM1.02	PRDI (positive regulatory domain I element) binding factor 1	375	0.887	tgacagtGA AAgcagat
V$GATA1.03	GATA-binding factor 1	385	0.953	agcaGAT Atgttc

APPENDIX C

CHAPTER 5: SUPPLEMENTAL INFORMATION[A]

[A]Alexandra B. Bentz, Chad Niederhuth, Laura Carruth, and Kristen J. Navara. To be submitted to *Current Biology*.

SUPPLEMENTAL EXPERIMENTAL METHODS

Mass and tarsus growth

We measured mass with a digital scale to 0.01g on hatch day (d1) and mass and tarsus (with digital calipers to 0.01mm) on days 2, 5, 8, 11, 12, and 14 post-hatch. We analyzed mass and tarsus using a repeated measures mixed effects model with either mass or tarsus as the dependent variable and treatment, age (in days), sex, egg number, brood size, and the interaction between treatment and day along with individual ID nested within natal cage ID as the random effect. For mass, the interaction between treatment and day was significant ($F_{6,275} = 2.70$, $p = 0.015$; Fig. S5.1A) while sex, egg number, and brood size were not significant (all $p > 0.062$). For tarsus, only day was significant ($F_{1,227} = 1216.81$, $p < 0.0001$; Fig. S5.1B) while treatment, sex, egg number, brood size, and the interaction between treatment and day were not significant (all $p > 0.10$). We further tested if mass and tarsus at 50 days post-hatch (approx. date of maturity) was influenced by treatment while accounting for sex and including natal cage ID as a random effect. Neither mass nor tarsus differed by treatment or sex at 50 days post-hatch (all $p > 0.19$).

Begging rate

We measured begging rate on day 5 post-hatch (prior to their eyes opening) by removing the nest from the cage and placing it on a heating pad for 1hr to ensure all nestlings were motivated to beg during a videotaped 5 min trial. During the trial, the researcher gently tapped the nestling on the bill every 3 s after the nestling stopped begging or after the previous stimulus if the chick did not beg (Gilbert et al. 2006). We recorded the number of seconds a nestling gaped during each begging bout and calculated the average across all begging bouts as the

average begging rate. We also measured the mass of each nestling before the nest was removed from the natal cage and then after the begging trial to calculate how much mass was lost during the food deprivation period. We used average begging rate as the dependent variable and treatment, sex, egg number, brood size, and mass loss during begging trial with natal cage as the random effect. Only treatment was significant ($F_{1,22} = 4.19$, $p = 0.05$; Fig. S5.2A) with nestlings from testosterone-injected eggs begging longer on average (7.4s +/- 0.7) than control nestlings (6.1 +/- 0.5); sex, egg number, brood size, and mass loss were not significant (all $p > 0.09$).

Bill maturation

On 50 days post-hatch each individual's bill was photographed from the front, left, and right. Images were uploaded to GIMP 2.6.12 to count the number of pixels that were black and the number that were in the total area. We determined the percentage of the bill that was still black in each of the three images and averaged it for each individual. The percentage of the bill that was black was the dependent variable with treatment, sex, and egg number as independent variables with natal cage as the random effect; however, none of the variables were significant (all $p > 0.20$; Fig. S5.2B).

Digit ratio

After sexual maturity was reached, digit length was measured on both feet using electronic digital calipers to the nearest 0.01mm and the ratio of the 2nd to the 4th digit (2D:4D) was calculated for both feet (Lombardo et al. 2008). The 2D:4D ratio was the dependent variable with treatment, sex, and egg number as independent variables with natal cage as the random

effect. Neither the left 2D:4D (Fig. S5.2C) or right 2D:4D (Fig. S5.2D) were affected by treatment, sex, or egg number (all $p > 0.20$).

Plasma testosterone

We collected trunk blood from the subset of individuals that were decapitated 30 min after the start of a conspecific intrusion. Blood was kept on ice until whole blood could be centrifuged to collect plasma which was stored at -20^{0}C. Extraction and radioimmunoassay of plasma testosterone was performed in one set following procedures described by Wingfield & Farner (1975). Average recovery rate for plasma testosterone was 94%. Plasma testosterone was the dependent variable with treatment, sex, and egg number as independent variables with natal cage and adult cage as random effects. Treatment was significant as birds from testosterone-injected eggs had significantly lower plasma testosterone values ($F_{1,14} = 5.18$, $p = 0.04$; Fig. S5.3). Egg number was also significant ($\beta = -0.62$; $F_{1,14} = 12.02$, $p = 0.004$); plasma testosterone decreased with egg number. Plasma testosterone did not differ by sex ($p = 0.06$).

ADDITIONAL TABLES AND FIGURES

Table S5.1. Most overrepresented biological processes from the list of genes differentially expressed between offspring from testosterone-injected or control eggs.

Sample	GO.ID	Term	Ann-otated	Sign-ificant	Exp-ected	p-value
Female Hypothalamus	GO:0009611	response to wounding	102	4	0.34	0.00019
	GO:0009072	aromatic amino acid family metabolic pro...	16	2	0.05	0.00214
	GO:0006082	organic acid metabolic process	368	5	1.21	0.00413
	GO:0034260	negative regulation of GTPase activity	2	1	0.01	0.00688
	GO:0032011	ARF protein signal transduction	13	2	0.04	0.00790
	GO:0033615	mitochondrial proton-transporting ATP sy...	1	1	0	0.00935
Female Nucleus Taeniae	GO:0006029	proteoglycan metabolic process	31	6	0.51	0.00014
	GO:0070314	G1 to G0 transition	3	2	0.05	0.00031
	GO:0040036	regulation of fibroblast growth factor r...	14	4	0.23	0.00031
	GO:0050748	negative regulation of lipoprotein metab...	3	2	0.05	0.00054
	GO:0050654	chondroitin sulfate proteoglycan metabol...	16	5	0.26	0.00075
	GO:0045935	positive regulation of nucleobase-contai...	622	14	10.2	0.00129
	GO:0010628	positive regulation of gene expression	631	17	10.35	0.00130
	GO:0090287	regulation of cellular response to growt...	94	7	1.54	0.00140
	GO:0050746	regulation of lipoprotein metabolic proc...	4	2	0.07	0.00164
	GO:0009100	glycoprotein metabolic process	127	6	2.08	0.00287
	GO:0030204	chondroitin sulfate metabolic process	13	4	0.21	0.00298
	GO:0007010	cytoskeleton organization	365	11	5.98	0.00311
	GO:0006023	aminoglycan biosynthetic process	22	3	0.36	0.00316
	GO:0044699	single-organism process	5533	107	90.71	0.00326
	GO:0010035	response to inorganic substance	80	7	1.31	0.00368
	GO:0006790	sulfur compound metabolic process	83	5	1.36	0.00385
	GO:0031099	regeneration	4	2	0.07	0.00397
	GO:0055075	potassium ion homeostasis	3	2	0.05	0.00401
	GO:0040007	growth	264	11	4.33	0.00404
	GO:0010646	regulation of cell communication	940	28	15.41	0.00421
	GO:0022610	biological adhesion	430	15	7.05	0.00455
	GO:0030166	proteoglycan biosynthetic proc.	18	4	0.3	0.00456

Sample	GO.ID	Term	Ann-otated	Sign-ificant	Exp-ected	Fisher
Female Nucleus Taeniae cont.	GO:0045599	negative regulation of fat cell differen...	22	3	0.36	0.00462
	GO:0044744	protein targeting to nucleus	98	5	1.61	0.00474
	GO:0023051	regulation of signaling	949	28	15.56	0.00506
	GO:0044344	cellular response to fibroblast growth f...	39	5	0.64	0.00543
	GO:0042246	tissue regeneration	4	2	0.07	0.00620
	GO:0070201	regulation of establishment of protein l...	158	7	2.59	0.00656
	GO:0071774	response to fibroblast growth factor	39	5	0.64	0.00663
	GO:1903530	regulation of secretion by cell	126	7	2.07	0.00676
	GO:0042445	hormone metabolic process	52	4	0.85	0.00735
	GO:0044763	single-organism cellular process	4776	91	78.3	0.00746
	GO:0033002	muscle cell proliferation	43	4	0.7	0.00790
	GO:0008589	regulation of smoothened signaling pathw...	32	4	0.52	0.00795
	GO:0051049	regulation of transport	400	14	6.56	0.00808
	GO:0007267	cell-cell signaling	351	14	5.75	0.00908
	GO:0044272	sulfur compound biosynthetic process	32	3	0.52	0.00990
Male hypothalamus	GO:0010604	positive regulation of macromolecule met...	954	54	39.47	0.00011
	GO:0051254	positive regulation of RNA metabolic pro...	572	38	23.67	0.00013
	GO:0009891	positive regulation of biosynthetic proc...	648	44	26.81	0.00013
	GO:0050748	negative regulation of lipoprotein metab...	3	3	0.12	0.00014
	GO:0007154	cell communication	2577	158	106.6	0.00016
	GO:0050890	cognition	9	3	0.37	0.00019
	GO:0009190	cyclic nucleotide biosynthetic process	59	12	2.44	0.00021
	GO:1903508	positive regulation of nucleic acid-temp...	566	38	23.42	0.00022
	GO:0044708	single-organism behavior	13	5	0.54	0.00024
	GO:0045893	positive regulation of transcription	DN...	566	38	23.42
	GO:0033688	regulation of osteoblast proliferation	13	5	0.54	0.00026
	GO:0061351	neural precursor cell proliferation	21	6	0.87	0.00029
	GO:0048519	negative regulation of biological proces...	1405	83	58.13	0.00034
	GO:0048523	negative regulation of cellular process	1302	76	53.87	0.00036

Sample	GO.ID	Term	Ann-otated	Sign-ificant	Exp-ected	Fisher
Male Hypothalamus cont.	GO:0050746	regulation of lipoprotein metabolic proc...	4	3	0.17	0.00039
	GO:0046390	ribose phosphate biosynthetic process	121	13	5.01	0.00045
	GO:0033687	osteoblast proliferation	15	5	0.62	0.00046
	GO:0010557	positive regulation of macromolecule bio...	615	38	25.44	0.00052
	GO:0040011	locomotion	365	29	15.1	0.00055
	GO:2000177	regulation of neural precursor cell prol...	15	5	0.62	0.00063
	GO:0048167	regulation of synaptic plasticity	47	10	1.94	0.00063
	GO:0048518	positive regulation of biological proces...	1585	90	65.58	0.00068
	GO:0042737	drug catabolic process	2	2	0.08	0.00071
	GO:0051179	localization	2245	120	92.88	0.00076
	GO:0032502	developmental process	908	57	37.57	0.00082
	GO:0080090	regulation of primary metabolic process	1911	90	79.06	0.00090
	GO:0007165	signal transduction	2422	145	100.2	0.00090
	GO:0048522	positive regulation of cellular process	1425	80	58.96	0.00097
	GO:0072522	purine-containing compound biosynthetic ...	127	13	5.25	0.00105
	GO:1902680	positive regulation of RNA biosynthetic ...	566	38	23.42	0.00105
	GO:0044707	single-multicellular organism process	829	59	34.3	0.00109
	GO:0007010	cytoskeleton organization	365	20	15.1	0.00127
	GO:0071241	cellular response to inorganic substance	22	6	0.91	0.00129
	GO:0050804	modulation of synaptic transmission	75	13	3.1	0.00136
	GO:0032879	regulation of localization	591	45	24.45	0.00158
	GO:0071804	cellular potassium ion transport	46	8	1.9	0.00174
	GO:0007215	glutamate receptor signaling pathway	29	7	1.2	0.00200
	GO:0045578	negative regulation of B cell differenti...	2	2	0.08	0.00202
	GO:0050768	negative regulation of neurogenesis	2	2	0.08	0.00246
	GO:0042981	regulation of apoptotic process	429	26	17.75	0.00249
	GO:0010646	regulation of cell communication	940	67	38.89	0.00261
	GO:0010629	negative regulation of gene expression	467	27	19.32	0.00269
	GO:0006182	cGMP biosynthetic process	17	6	0.7	0.00314

Sample	GO.ID	Term	Ann-otated	Sign-ificant	Exp-ected	Fisher
Male Hypothalamus cont.	GO:0017144	drug metabolic process	3	2	0.12	0.00340
	GO:0034762	regulation of transmembrane transport	86	12	3.56	0.00342
	GO:0034220	ion transmembrane transport	205	21	8.48	0.00347
	GO:0060255	regulation of macromolecule metabolic pr...	1927	86	79.73	0.00380
	GO:0023051	regulation of signaling	949	67	39.26	0.00415
	GO:0006464	cellular protein modification process	1675	64	69.3	0.00437
	GO:2000113	negative regulation of cellular macromol...	454	26	18.78	0.00438
	GO:0072521	purine-containing compound metabolic pro...	214	15	8.85	0.00446
	GO:0051241	negative regulation of multicellular org...	250	23	10.34	0.00454
	GO:0010605	negative regulation of macromolecule met...	683	36	28.26	0.00467
	GO:0050728	negative regulation of inflammatory resp...	28	5	1.16	0.00481
	GO:0051253	negative regulation of RNA metabolic pro...	411	25	17	0.00487
	GO:0046068	cGMP metabolic process	22	6	0.91	0.00494
	GO:0010558	negative regulation of macromolecule bio...	465	27	19.24	0.00505
	GO:0030001	metal ion transport	338	29	13.98	0.00510
	GO:0060687	regulation of branching involved in pros...	7	3	0.29	0.00532
	GO:0052652	cyclic purine nucleotide metabolic proce...	55	11	2.28	0.00540
	GO:0045165	cell fate commitment	64	10	2.65	0.00566
	GO:0001655	urogenital system development	45	8	1.86	0.00591
	GO:0033690	positive regulation of osteoblast prolif...	7	3	0.29	0.00593
	GO:0072073	kidney epithelium development	23	5	0.95	0.00602
	GO:0033058	directional locomotion	2	2	0.08	0.00611
	GO:0032501	multicellular organismal process	1058	60	43.77	0.00640
	GO:0051402	neuron apoptotic process	77	9	3.19	0.00663
	GO:0032774	RNA biosynthetic process	1343	58	55.56	0.00698
	GO:0001822	kidney development	29	6	1.2	0.00734
	GO:0050919	negative chemotaxis	10	3	0.41	0.00771
	GO:0071392	cellular response to estradiol stimulus	3	2	0.12	0.00839
	GO:0070997	neuron death	80	9	3.31	0.00853
	GO:0045934	negative regulation of nucleobase-contai...	457	25	18.91	0.00878

Sample	GO.ID	Term	Ann-otated	Sign-ificant	Exp-ected	Fisher
Male Hypothalamus cont.	GO:0051049	regulation of transport	400	32	16.55	0.00940
	GO:0044271	cellular nitrogen compound biosynthetic ...	1874	76	77.53	0.00942
	GO:0006164	purine nucleotide biosynthetic process	123	13	5.09	0.00948
	GO:0009260	ribonucleotide biosynthetic process	121	13	5.01	0.00990
Male Nucleus Taeniae	GO:0048525	negative regulation of viral process	8	2	0.05	0.0017
	GO:0044707	single-multicellular organism process	829	13	4.74	0.0018
	GO:0001709	cell fate determination	7	2	0.04	0.0019
	GO:0051704	multi-organism process	104	4	0.6	0.0029
	GO:0007272	ensheathment of neurons	12	2	0.07	0.0029
	GO:0010628	positive regulation of gene expression	631	9	3.61	0.0030
	GO:0032502	developmental process	908	12	5.2	0.0044
	GO:0060341	regulation of cellular localization	153	4	0.88	0.0044
	GO:0032501	multicellular organismal process	1058	13	6.05	0.0055
	GO:0065007	biological regulation	4461	35	25.53	0.0060
	GO:0050789	regulation of biological process	4286	34	24.52	0.0061
	GO:0040037	negative regulation of fibroblast growth...	8	2	0.05	0.0064
	GO:0032387	negative regulation of intracellular tra...	28	2	0.16	0.0066
	GO:0031056	regulation of histone modification	32	2	0.18	0.0070
	GO:0040019	positive regulation of embryonic develop...	17	2	0.1	0.0084
	GO:1902275	regulation of chromatin organization	38	2	0.22	0.0087
	GO:0001759	organ induction	17	2	0.1	0.0092

Table S5.2. Behavioral genes associated with aggression that are significantly differentially expressed between individuals from control and testosterone-injected eggs. Significance was cut-off at $p < 0.01$ and were corrected with a Benjamini–Hochberg procedure.

Sample	Gene_ID	Gene	Control Mean (FPKM[1])	Testosterone Mean (FPKM[1])	Log2 fold change	Adj. p-value
Male Hypothalamus	ENSTGUG00000000003	EGR1	82.78	275.95	1.74	5E-05
	ENSTGUG00000000359	CRHR2	14.83	52.61	1.83	5E-05
	ENSTGUG00000001436	HTR1D	5.34	20.31	1.93	5E-05
	ENSTGUG00000001972	MC4R	24.54	5.75	-2.09	0.0001
	ENSTGUG00000002234	PNMT	66.58	8.63	-2.95	5E-05
	ENSTGUG00000004743	BDNF	20.15	65.62	1.70	5E-05
	ENSTGUG00000005314	NPY2R	26.38	70.25	1.41	5E-05
	ENSTGUG00000005715	RAPGEF2	33.87	71.11	1.07	5E-05
	ENSTGUG00000006869	SSTR5	4.24	21.61	2.35	5E-05
	ENSTGUG00000007457	HTR2C	19.61	63.55	1.70	5E-05
	ENSTGUG00000007930	DDC	12.15	2.17	-2.48	0.001
	ENSTGUG00000010372	NOS1	23.39	7.41	-1.66	5E-05
	ENSTGUG00000004054	CRHBP	78.19	180.98	1.21	0.00015
Female Hypothalamus	ENSTGUG00000007300	TPH2	22.26	3.84	-2.53	0.0003
	ENSTGUG00000009295	TH	36.54	4.95	-2.88	5E-05
Female Nucleus Taeniae	ENSTGUG00000004054	CRHBP	38.65	96.13	1.31	0.0004
	ENSTGUG00000002810	NPY	879.08	348.27	-1.34	0.0002

[1]Fragments Per Kilobase of transcript per Million mapped reads

Table S5.3. Linear regression models for the relationship between average aggression score and scaled log(1+FPKM) data (Z-score). For each analyis, n = 6 individuals and Benjamini-Hochberg procedures were used to adjust p-values, significant values are in bold.

Sample	Gene	β	F	p-value	Adj. p-value
Male Hypothalamus	EGR1	1.48	2.59	0.18	0.26
	CRHR2	3.46	13.05	0.02	0.07
	HTR1D	2.67	3.33	0.14	0.22
	MC4R	-3.63	21.51	0.01	**0.04**
	PNMT	-3.69	26.60	0.01	**0.04**
	BDNF	3.21	7.73	0.05	0.53
	NPY2R	3.38	10.76	0.03	0.07
	RAPGEF2	3.24	8.06	0.05	0.09
	SSTR5	3.34	9.93	0.03	0.07
	HTR2C	3.34	9.93	0.03	0.07
	DDC	-3.65	22.35	0.01	**0.04**
	NOS1	-3.67	24.08	0.01	**0.04**
	CRHBP	1.58	0.76	0.43	0.49
Female Hypothalamus	TPH2	-0.72	0.90	0.40	0.49
	TH	-0.31	0.14	0.73	0.73
Female Nucleus Taeniae	CRHBP	1.20	4.19	0.11	0.19
	NPY	-0.79	1.15	0.34	0.44

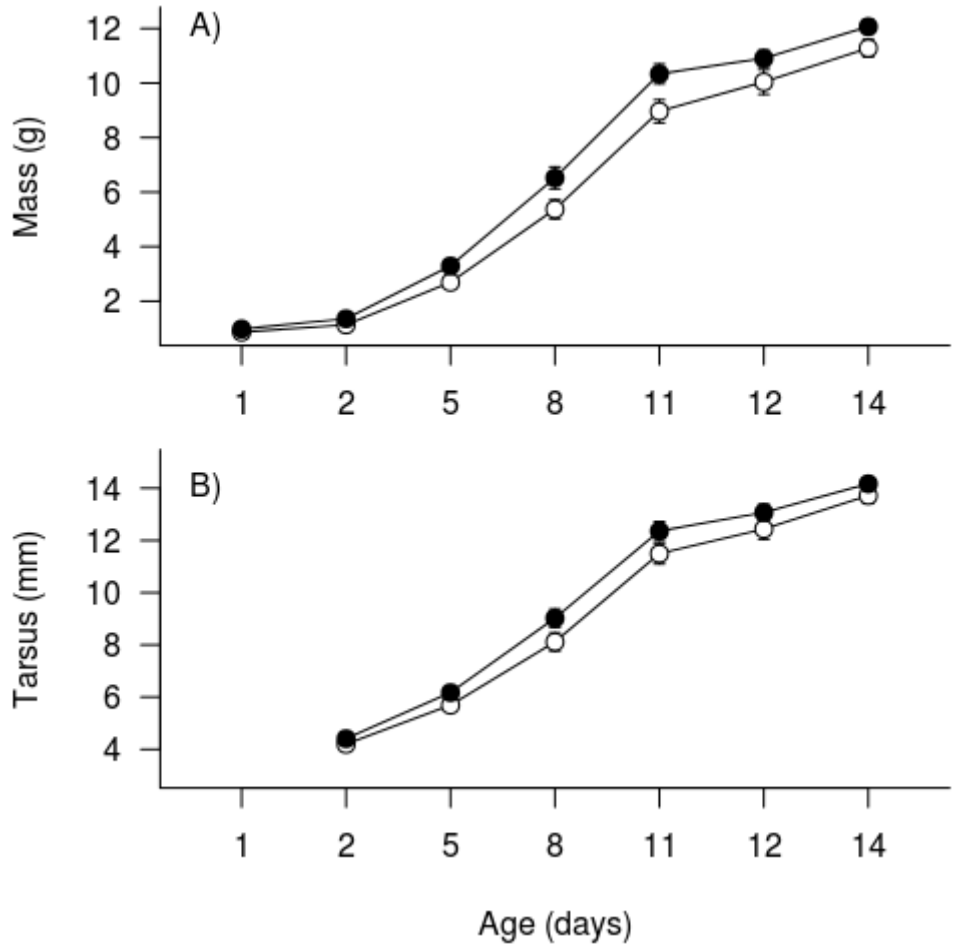

Figure S5.1. Relationship between A) mass (in g) and B) tarsus (in mm) change over time and treatment (control, open circles, or testosterone, closed circles, egg injections). Errorbars denote SE.

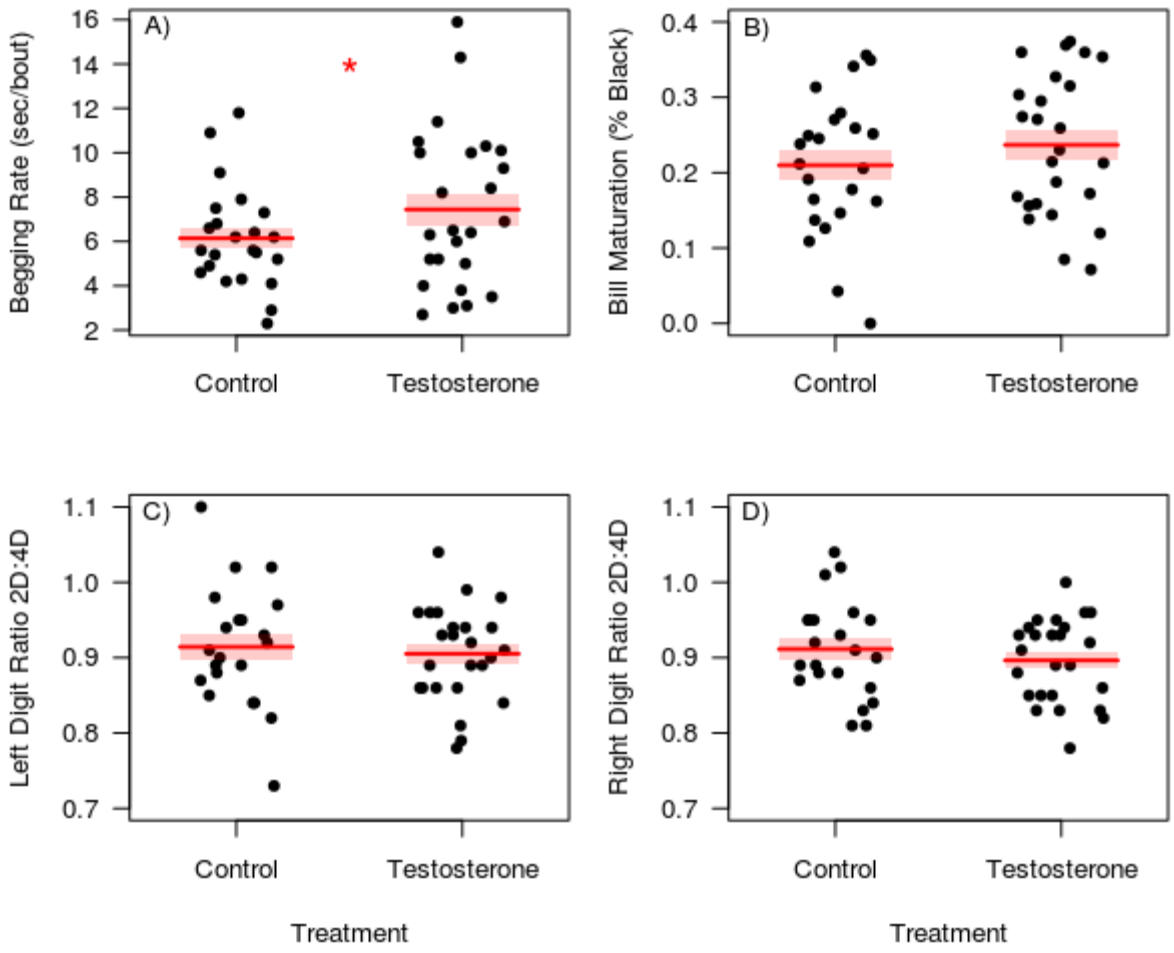

Figure S5.2. Relationship between A) begging rate, B) bill maturation, C) left 2nd to 4th digit ratio (2D:4D), and D) right 2D:4D and treatment (control or testosterone injected eggs). The red line represents the mean and the shaded area is SE. Asterisks denote significance ($p < 0.05$) based on linear mixed effects models.

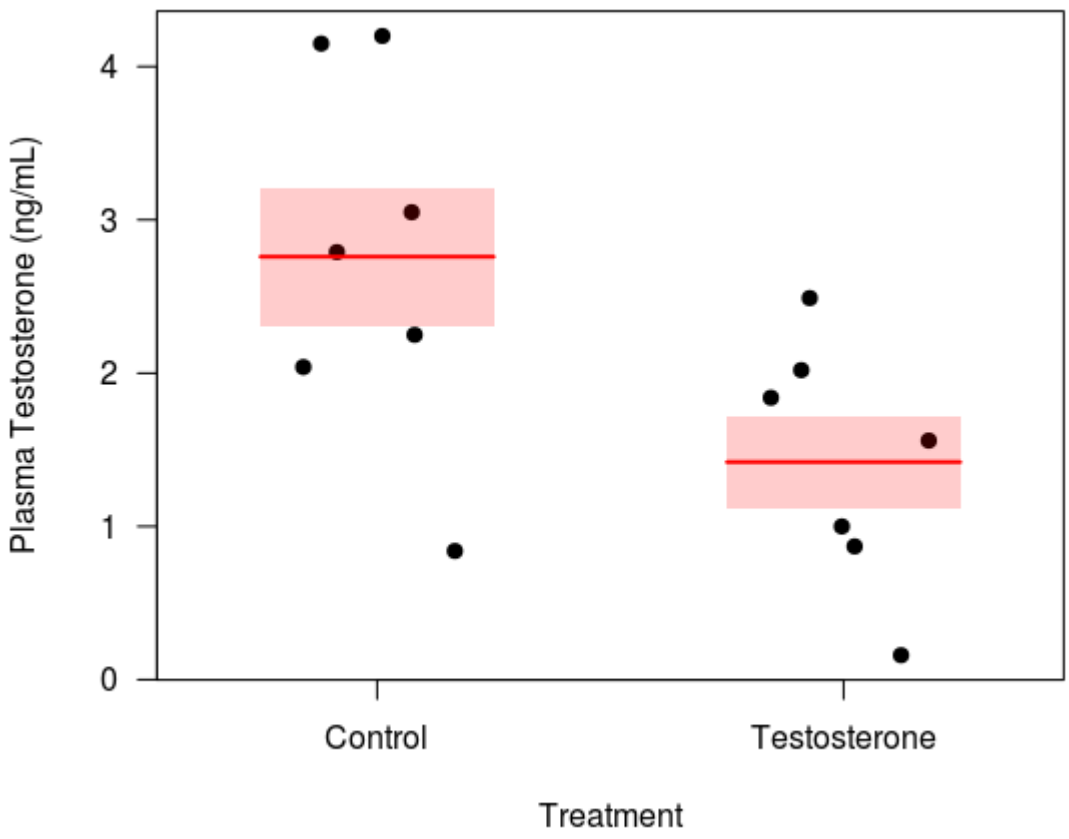

Figure S5.3. Relationship between plasma testosterone (ng/mL) in male and female zebra finches 30 min after a conspecific intrusion and treatment (control or testosterone injected eggs). The red line represents the mean and the shaded area is SE.

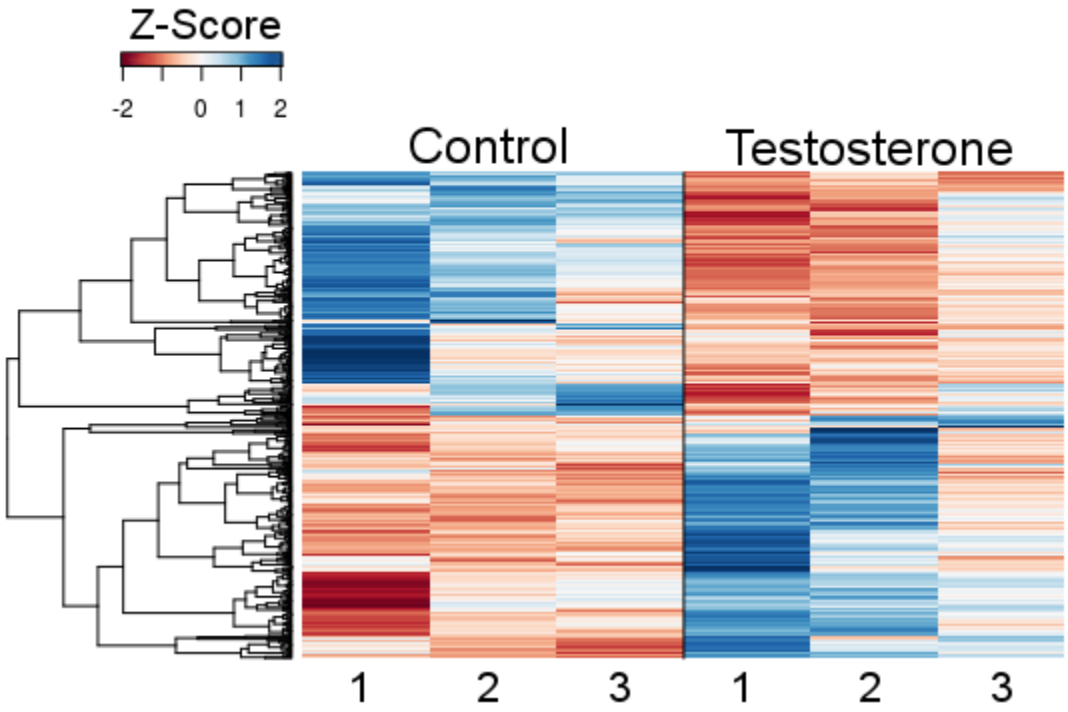

Figure S5.4. Differentially expressed genes (n = 612; $p < 0.01$) in the hypothalamus of control (n = 3) and testosterone (n = 3) males. Blue = upregulated and red = downregulated compared to the mean value of a gene across all samples. Log(1+FPKM) data are scaled (Z-score) so that units of change are standard deviations from the mean. Genes were clustered using Pearson correlation to construct the dendrogram.

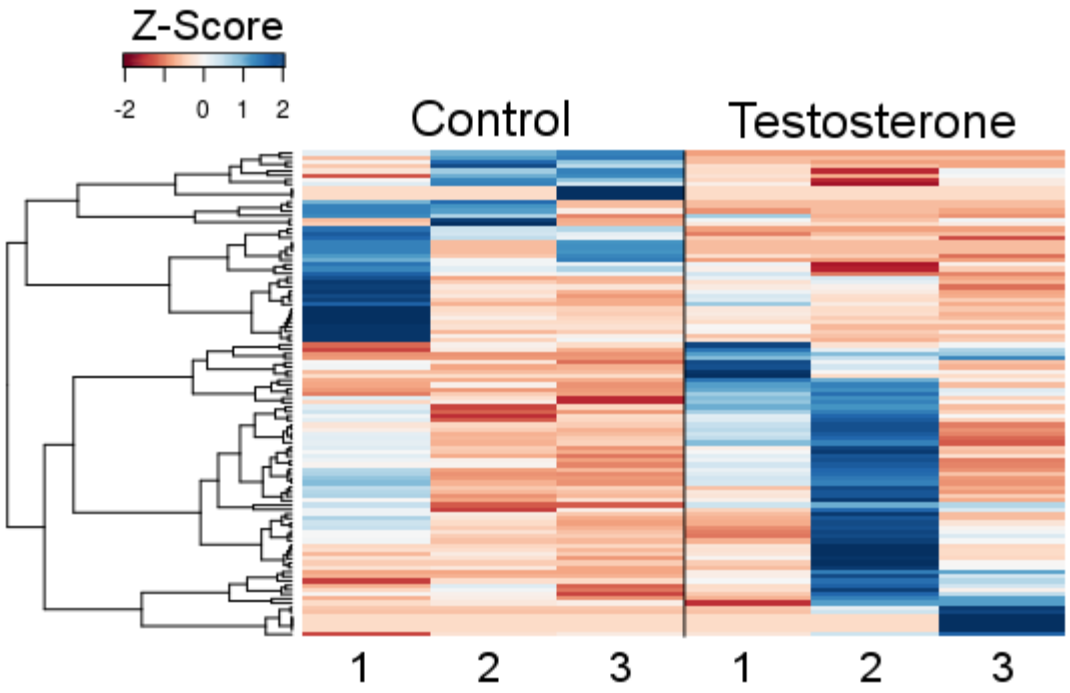

Figure S5.5. Differentially expressed genes (n = 109; p<0.01) in the nuecleus taeniae of control (n = 3) and testosterone (n = 3) males. Blue = upregulated and red = downregulated compared to the mean value of a gene across all samples. Log(1+FPKM) data are scaled (Z-score) so that units of change are standard deviations from the mean. Genes were clustered using Pearson correlation to construct the dendrogram.

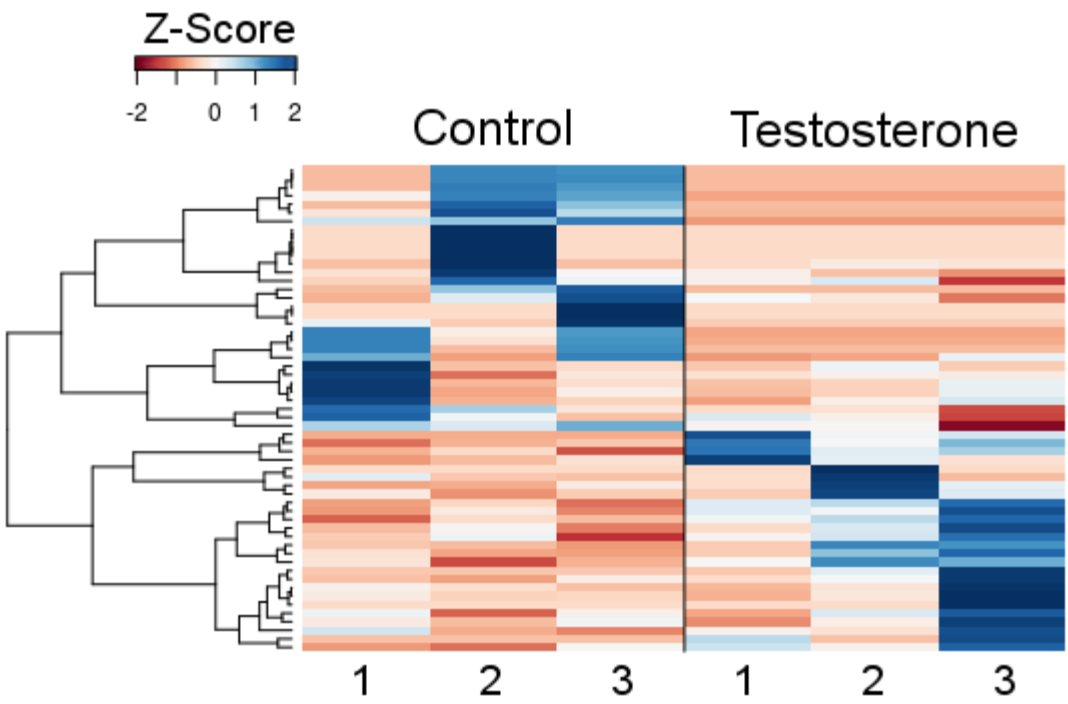

Figure S5.6. Differentially expressed genes (n = 57; p<0.01) in the hypothalamus of control (n = 3) and testosterone (n = 3) females. Blue = upregulated and red = downregulated compared to the mean value of a gene across all samples. Log(1+FPKM) data are scaled (Z-score) so that units of change are standard deviations from the mean. Genes were clustered using Pearson correlation to construct the dendrogram.

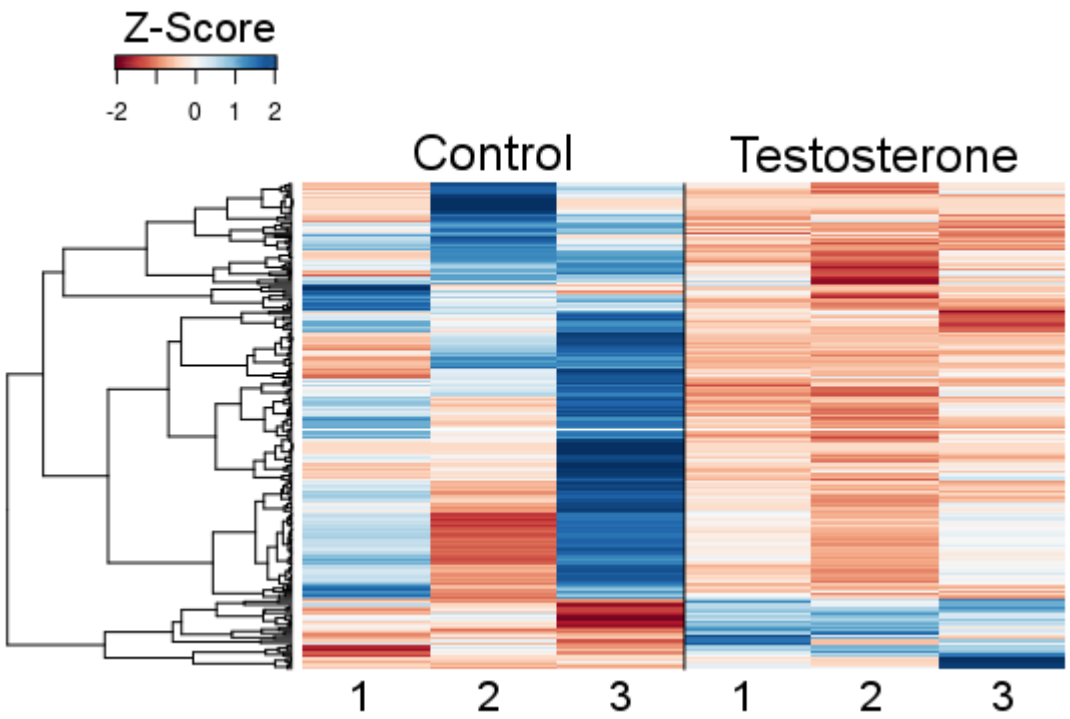

Figure S5.7. Differentially expressed genes (n = 229; $p<0.01$) in the nucleus taeniae of control (n = 3) and testosterone (n = 3) females. Blue = upregulated and red = downregulated compared to the mean value of a gene across all samples. Log(1+FPKM) data are scaled (Z-score) so that units of change are standard deviations from the mean. Genes were clustered using Pearson correlation to construct the dendrogram.

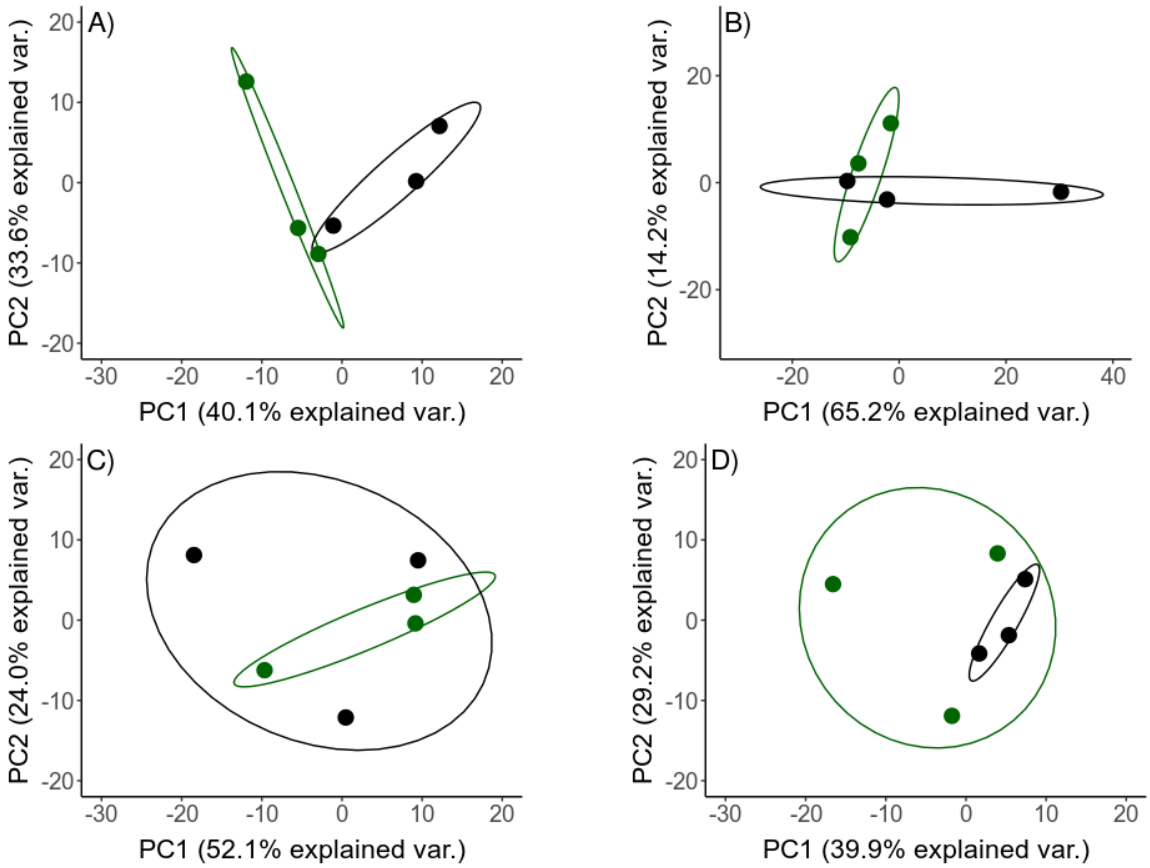

Figure S5.8. Biplots of Principal Component Analysis (PCA) of all annotated genes (n = 8,422 genes) in the A) male hypothalamus, B) male nucleus taeniae, C) female hypothalamus, and D) female nucleus taeniae based on egg treatment (control, green circles, or testosterone, black circles). Ellipses represent 95% confidence intervals for each treatment and PCA was performed with log(1+FPKM) data.