

Phenotypic plasticity in response to breeding density in tree swallows: An adaptive maternal effect?



Alexandra B. Bentz^{a,*}, Kristen J. Navara^b, Lynn Siefferman^a

^a Biology Department, Appalachian State University, 572 Rivers St., Boone, NC 28608, USA

^b Poultry Science Department, University of Georgia, 203 Poultry Science Bldg., Athens, GA 30602, USA

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ABSTRACT

Territorial animals breeding in high-density environments are more likely to engage in aggressive competition with conspecifics for resources necessary for reproduction. In many avian species, increased competition among breeding females results in increased testosterone concentrations in egg yolks. Generally, elevated yolk testosterone increases nestling growth, competitive behaviors, and bold behavioral traits. However, few studies provide an environmental context with which to examine the potential adaptive benefits of these phenotypic changes. In this study, tree swallow (*Tachycineta bicolor*) breeding density was altered to modify levels of social competition and yolk testosterone. We measured nestling growth, competitive ability, and breathing rate in response to a stressor using a partial cross-foster design. Females breeding at high-density experienced more aggressive, competitive interactions and their eggs had higher testosterone concentrations. Nestlings that hatched in high-density environments grew faster and displayed more competitive behaviors and a higher breathing rate response to a stressor regardless of post-hatching density. Our study demonstrates that phenotypic plasticity occurs in response to yolk testosterone variation resulting from different breeding densities. These findings suggest that naturally-induced maternal effects prepare offspring for competitive environments, supporting the idea that maternal effects are adaptive.

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Introduction

Animals display aggressive behaviors toward conspecifics for numerous reasons, a common reason being competition for breeding resources. Territorial and monogamous female songbirds that are better at competing benefit by acquiring and maintaining breeding territories and pairing with a monogamous mate (Dunn and Hannon, 1991; Rosvall, 2008). In addition, several studies show a positive relationship between aggression among females competing for breeding resources and concentrations of androgens (e.g., testosterone) in their egg yolks (Hargitai et al., 2009; Mazuc et al., 2003; Navara et al., 2006b; Whittingham and Schwabl, 2001). Environmentally-induced variation in egg hormones is a classic example of a maternal effect, a non-genetic mechanism in which conditions females experience influence offspring phenotype (Groothuis et al., 2005; Wolf and Wade, 2009). Whether or not maternal effects are beneficial to females competing for resources or their offspring has yet to be conclusively shown.

The effects of increased yolk testosterone on offspring phenotype are best documented in terms of growth and behaviors. Artificially

increased yolk testosterone increases nestling growth (Lipar and Ketterson, 2000; Muller et al., 2007, 2009; Navara et al., 2005, 2006a; Pilz et al., 2004; Schwabl, 1996; but see Andersson et al., 2004; Rubolini et al., 2006; Schwabl et al., 2012) and metabolic rate (Nilsson et al., 2011; Tobler et al., 2007). Artificially enhanced yolk testosterone also causes more competitive behaviors in nestlings, such as increased begging rate and duration (Eising and Groothuis, 2003; von Engelhardt et al., 2006) and territorial behavior (Muller et al., 2009). Furthermore, nestlings exposed to high levels of yolk testosterone display enhanced social dominance as adults (Eising et al., 2006; Partecke and Schwabl, 2008; Strasser and Schwabl, 2004). Nestlings from testosterone-injected eggs also display bolder behaviors (i.e., fewer distress vocalizations and quickly approach novel objects; Daisley et al., 2005). In general, nestlings exposed to high yolk testosterone have a more “active coping” style, which is characterized by high competitive ability, bold behaviors, and greater sympathetic reactivity as opposed to those with a more “passive coping” style defined by behavioral inhibition (Koolhaas et al., 1999, 2010). Unfortunately, few studies, if any, have examined the influence of yolk hormones on autonomic nerve responses (i.e., sympathetic and parasympathetic reactivity), despite their importance in determining how individuals cope with challenges (Chichinadze and Chichinadze, 2008; Koolhaas et al., 1999, 2010).

Environmentally-induced variation in yolk hormone provisioning likely serves an adaptive purpose. Maternal effects may be an adaptive means by which a female changes the phenotype of her offspring in

* Corresponding author.

E-mail addresses: alexanda.b.bentz@gmail.com (A.B. Bentz), knava@uga.edu (K.J. Navara), sieffermanlm@appstate.edu (L. Siefferman).

¹ Present address: Department of Biological Sciences, Auburn University, 101 Life Sciences Building, Auburn, AL 36849, USA.

preparation for a given environment (Carere and Balthazart, 2007; Groothuis et al., 2005; Mousseau and Fox, 1998; Schwabl, 1997). Breeding density, for example, is a prominent environmental factor that can increase levels of aggressive competition (Male et al., 2006) and circulating testosterone in females (Smith et al., 2005). A positive relationship between density and yolk testosterone is seen in European starlings (*Sturnus vulgaris*; Pilz and Smith, 2004), tree swallows (*Tachycineta bicolor*; Whittingham and Schwabl, 2001), house sparrows (*Passer domesticus*; Mazuc et al., 2003; Schwabl, 1997), American coots (*Fulica americana*; Reed and Vleck, 2001), and collard flycatchers (*Ficedula albicollis*; Hargitai et al., 2009). The phenotypic changes associated with high yolk testosterone (i.e., increased growth and a more active coping style) would certainly appear to be beneficial in high breeding densities. However, to date, most studies have investigated the influence of maternal hormones on offspring by artificially increasing yolk testosterone, which lacks the ability to show that specific phenotypes arise in response to an environment. Thus, the adaptive value of phenotypes derived from this maternal effect can only be assumed in the absence of an environmental context.

Here, we tested for correlations between breeding density and yolk testosterone as well as between yolk testosterone and offspring growth. Following this, we then experimentally manipulated breeding density and measured yolk testosterone concentrations. Yolk testosterone was expected to positively correlate with density because of increased numbers of competitive interactions (Whittingham and Schwabl, 2001). We then measured subsequent phenotypic differences in growth, competitive ability in the nest, and breathing rate in response to handling stress in the altricial offspring of tree swallows. Breathing rate is commonly used in avian studies to measure responsiveness to handling stress and has been linked to coping style (Carere and van Oers, 2004; Fucikova et al., 2009; van den Brink et al., 2012). Furthermore, by incorporating a cross-foster design, we tested the relative importance of pre-natal breeding density versus post-natal environment in influencing offspring phenotype. Therefore, this study tests whether a naturally-induced maternal effect can act as a proximate mechanism influencing the development of offspring phenotype in anticipation of a high-density environment. If higher yolk testosterone in eggs laid at high-density cause nestling phenotypes to be characterized by faster growth and a more active coping style, then this maternal effect is likely an adaptive mechanism to prepare offspring for competitive environments.

Methods

Study species

We studied a natural population of tree swallows, a socially-monomogamous passerine, in Watauga County, North Carolina (36°12'41"N, 81°40'7"W) between April and July in 2009 and 2011. Our study sites are characterized by hayfields and pastures and are no more than 5 km apart. Tree swallows readily accept nest boxes (Robertson et al., 1992) and are particularly appropriate for testosterone-related research because females aggressively compete for their cavities (Rosvall, 2008). This species breeds in a variety of nesting densities and the number of agonistic interactions between females is positively related to nesting density (Male et al., 2006). Furthermore, tree swallows do not vary yolk androgen concentrations within a clutch, but rather between clutches in response to environmental factors, such as breeding density (Whittingham and Schwabl, 2001). All animals were treated in accordance with the Appalachian State University Institutional Animal Care and Use Committee (IACUC).

Correlational study

In 2009, we placed nest boxes randomly throughout our study sites to determine natural breeding densities and the potential for the

influence of breeding density on offspring traits. We recorded breeding density by delineating a 300 m radius around each nest box in ArcGIS v. 10 (ESRI, Redlands, CA) and recorded the proportion of active nests during each pair's breeding cycle. Tree swallows typically remain within 100–300 m of the breeding site when foraging, so a 300 m radius should encompass the area within which a female could contact a conspecific (McCarty and Winkler, 1999). Yolk testosterone concentrations were determined for each clutch by collecting the 3rd egg; eggs were collected prior to incubation to ensure that hormones measured were maternally-derived (Elf and Fivizzani, 2002). Eggs were then frozen at -20°C and yolk testosterone was measured via radioimmunoassay (see detailed methods below). Nestling growth rate was determined by measuring mass (± 0.1 g) on days 2, 5, 8, 11, and 14 post-hatch. Growth follows a sigmoidal pattern and normally begins to plateau after day 11. Therefore, growth rates were derived from the slope of a linear regression of nestling mass on days 2–11 post-hatch (Hinde et al., 2009) and averaged for each brood.

Density manipulation

In 2011, we manipulated breeding density creating high- and low-density sites with the intention of altering aggressive interactions among female tree swallows. Breeding densities were measured the same way as the 2009 correlational study (see detailed methods above). In the high-density site, 53 nest boxes were placed so that no box was > 12 m from the nearest nest box. All nest boxes (53) fit within each individual box's 300 m radius when measuring breeding density, allowing each box at a site to have the same potential density. In the low-density site, 50 nest boxes were placed so that no box was < 36 m apart and, on average, 10 nest boxes fit within the 300 m radius of each pair's nest box.

To confirm that our nest box manipulation affected the number of social interactions tree swallows experienced, we observed randomly selected pairs for vocal and physical interactions for 20 min once the pair had successfully built a nest. To determine total number of interactions, all physical and vocal interactions were combined (Male et al., 2006).

Parental measurements

In 2011, at the high- and low-density sites, we measured female aggression levels to determine if the number of aggressive interactions or yolk testosterone concentrations at the sites were a product of breeding density or inherent aggression of females that choose these sites. During the incubation stage, we presented pairs with a conspecific model and a playback of tree swallow chatter at their nest box for 5 min (i.e., simulated territorial intrusion) and counted how often pairs aggressively flew by, hovered, or attacked the model (Duckworth, 2010). Adults were given unique bands to aid in their identification. Parental feeding rates were measured during the competition trials for nestlings (see methods below) as the number of feeding visits per minute. We also captured each female and measured mass (± 0.1 g) and wing chord (± 0.1 mm) to create an index of female body condition using residuals of a regression of mass on wing length ($R^2 = 0.11$, $F_{1,51} = 6.28$, $p = 0.016$; Brown, 1996). Female age was estimated as second year (SY) or after-second year (ASY) using plumage coloration (Hussell, 1983).

To determine yolk hormone concentrations, we again collected the 3rd egg from each clutch prior to incubation and froze them at -20°C , and yolk testosterone and corticosterone were measured via radioimmunoassay (see detailed methods below). Yolk corticosterone was measured because previous studies link yolk corticosterone with breeding density (Love et al., 2008) and because corticosterone can negatively influence testosterone concentrations (Henriksen et al., 2011). The percentage of mass the yolk comprised in each egg was

also calculated as % yolk, a measure of egg quality (Hartmann and Wilhelmson, 2003; Ricklefs, 1984).

Radioimmunoassay of yolk

Yolk testosterone and corticosterone were extracted from homogenized yolk samples with a double ether extraction followed by liquid column chromatography according to methods described by Schwabl (1993). Briefly, 15 mg of yolk was weighed and vortexed with 1000 μ l 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 using a N_2 stream. The sample was reconstituted in 1.0 ml 10% ethyl acetate in isooctane and individual steroids were separated using celite column chromatography. Steroids were eluted in the following fractions: testosterone—10% ethyl acetate in isooctane, and corticosterone—50% ethyl acetate in isooctane. Testosterone and corticosterone were quantified using standard competitive-binding radioimmunoassays (using anti-testosterone and anti-corticosterone from MP Biomedicals, Solon, OH) as described in Wingfield and Farner (1975). All samples were done in one assay for each hormone. Average recoveries were 89.3% for testosterone and 85.5% for corticosterone. Intrassay variation was 2.97% for testosterone and 1.93% for corticosterone.

Nestling measurements

The pre-natal environment of offspring is influenced by maternal hormones, while post-natal environment is influenced by parental and environmental factors, yet both are expected to influence offspring phenotype. To account for this, in 2011, at the high- and low-density sites, nestling mass was measured on day 2 post-hatch (day 1 = hatch date) and nestlings of comparable size were exchanged between broods with identical hatch dates such that half of the nestlings from a low-density site brood were swapped with half of the nestlings from a high-density site brood ($n = 20$ nests). This allowed us to compare the phenotype of nestlings reared in the same environmental conditions, but that were prenatally exposed to different maternal breeding densities. This design separated the effects of pre-natal hormones and post-natal environment on nestling phenotype. Additional nestling mass (± 0.1 g) measurements were taken on days 5, 8, 11, and 14 post-hatch to quantify growth. Nestlings were identified by unique marks with a Sharpie® prior to day 8, at which point they were given unique bands.

In 2011, breathing rates of nestlings during handling stress were measured at age 14 days. We measured breathing rate of each nestling for a 1 min period in 15 s intervals immediately after being removed from the nest box. This was performed twice and repeatability was calculated for the average breathing rate in a 1 min period using the intraclass correlation coefficient (Lessells and Boag, 1987). We compared the first and last 15 s intervals using a *t*-test to determine if breathing rate changed over the 1 min period. Warmer air temperatures can increase respiration (Salt, 1964); accordingly, we tested effects of temperature. Air temperature significantly influenced average breathing rate ($R^2 = 0.08$, $F_{1,67} = 6.08$, $p = 0.02$); therefore, breathing rate response scores are residuals of a regression of average breathing rate on air temperature so that higher scores indicate a higher breathing rate for a given temperature.

Competitive ability of nestlings was measured using food competition trials conducted at age 14 days post-hatch (± 1 day) in 2011. Due to constraints placed on the parents by the design of the nest boxes, parents should preferentially feed offspring nearest the entrance to the box, thus, competition among offspring for this location is expected (Kolliker and Richner, 2004; McRae et al., 1993). We mounted small cameras adjusted for low light (HRC-20HEX; SCS, Inc., Montebello, NY, USA) within the nest boxes. The nest box was blocked for 1 h to ensure nestlings

were hungry and to allow parents to habituate to the presence of the camera. After this time, we re-opened the nest box and recorded parental provisioning for 1 h (trials began once parents started feeding). We quantified offspring begging intensity (i.e. begging posture; Pilz et al., 2004), begging frequency (beggings per feeding bout), and begging location in the nest (relative to nest entrance: center, far, left, right, and near; McRae et al., 1993). We used a Chi-square test to determine the ideal positions in the nest. The center and near locations were fed at more often than expected (number of feeds: far = 27, left = 10, right = 22, center = 99, near = 123 of 281 total feeds; $\chi^2 = 38.92$, $df = 4$, $p < 0.001$) and the percentage of a nestling's beggings in the center and near ("ideal") positions was calculated. A principal component of begging intensity (average begging posture), frequency (beggings per feeding bout), and location (percentage of beggings in ideal positions) was created to define an individual's competitive ability (Eigenvalue = 1.53, 50.89%; loading values: begging intensity = 0.74, frequency = 0.63, and location = 0.76). Therefore, nestlings with a higher competitive ability score begged more vigorously, begged more frequently, and occupied the ideal feeding spots for a greater percentage of begging bouts.

Statistical analyses

Data were tested for normality with Shapiro–Wilk tests (all $p > 0.06$); yolk corticosterone was non-normal and transformed with Log10 to approximate normality. SPSS (ver. 17.0; SPSS, Inc., Chicago, IL) was used to analyze data. All tests are two-tailed and all means are followed by standard error. A linear regression analysis was used to analyze the 2009 correlational data. A Chi-square analysis was used to determine if high- versus low-density sites were more likely to be used by SY or ASY females and *t*-tests were used to compare reproductive performance and condition of birds at high- and low-density sites. We used an ANCOVA to determine if number of natural aggressive interactions among adults and yolk testosterone (dependent variables) were affected by site density (independent variable) with inherent aggression scores as the covariate. No interactions were significant ($p > 0.1$) and were removed from the model.

General Linear Mixed Models were used to determine whether nestling behaviors (competitive ability and breathing rate response to a stressor) were influenced by pre- or post-natal site density. A Repeated Measures General Linear Mixed Model was used to test nestling growth using age (days 2, 5, 8, 11, and 14 post-hatch) as the repeated measure and nestling ID as the subject. The fixed effects tested in each model included: density manipulation (pre- and post-natal site), female age, and cross-foster status (whether they were raised by biological or foster parents). Additionally, we included nestling age as a fixed factor in the repeated measures model. Female age (all $\chi^2 > 0.49$, all $p > 0.48$) and cross-foster status (all $\chi^2 > -0.36$, all $p > 0.48$) were not significant in any models and were not included in subsequent analyses. The covariates in each model included hatch date, brood size, % yolk, yolk testosterone, and yolk corticosterone. Additionally, we included nestling mass at fledging and parental feeding rate as a covariate when testing nestling competitive ability, nestling mass at fledging when testing nestling breathing rate response, and feeding rate, begging frequency, and begging success (number of feeds received per times begged) when testing nestling growth. In all mixed models, multicollinearity among the predictor variables was tested for with a correlational matrix and for all variables $r < 0.7$. Random effects included were pre- and post-natal nest ID; however, random effects were excluded if they were determined non-significant by a Wald *Z* test or did not improve the model fit. In all cases, removal of random effects did not alter the significance of main effects.

The fit of each mixed model was tested using a Likelihood Ratio Test (LRT) to determine if random and main effects significantly improved the model fit and should be incorporated. The procedure included calculating the difference in the -2 log likelihood score between the initial model and the model plus or minus the effect being tested. We used

these results as a Chi-square value with df equal to the number of parameters changed between the two models (Pinheiro and Bates, 2000; West et al., 2007). We used both forward and backward model selection procedures to determine the best model fit. In all cases, both procedures resulted in the same model, therefore, only Chi-square values from forward selection procedures are presented; negative scores indicate a better model fit and positive scores indicate a worse model fit. Furthermore, we tested for interactions and none were significant (all $p > 0.10$).

We performed a Linear Regression analysis to determine if competitive ability conferred an advantage by influencing the percentage of feeds nestlings received. Moreover, because nestlings are chiefly competing with one another, we ranked nestling competitive ability within a nest and evenly split them into high and low competitive groupings. We then performed a Paired *t*-test to determine if competitive ability influenced proportion of feeds received in relation to competition with siblings, the results of which should be significant if nestlings within a nest are fed unevenly based on their competitive ability.

Results

Correlational study

Breeding density ranged from 4 to 19 pairs within a 300 m radius, with an average of 11 pairs. We documented a positive relationship between breeding density and yolk testosterone ($R^2 = 0.56$, $F_{1,9} = 10.31$, $p = 0.012$; Fig. 1) and between yolk testosterone and brood average growth rate ($R^2 = 0.81$, $F_{1,7} = 25.29$, $p = 0.002$; Fig. 2).

Density manipulation

The site designated as low density ultimately had an average of 6 ± 0.58 pairs within a 300 m radius and the site designated as high density had an average of 27 ± 1.01 pairs within a 300 m radius, which was significantly greater ($t_{1,18} = 31.76$, $p < 0.001$). Site density significantly influenced number of aggressive interactions, with more interactions occurring at the high-density site (4.46 ± 0.55 interactions) than the low-density site (2.06 ± 0.66 interactions; overall model: $F_{2,16} = 4.61$, $p = 0.03$; Table 1). Female age was not significantly different between the two sites ($\chi^2 = 2.92$, $df = 1$, $p = 0.09$) and the sites did not differ in timing of breeding, clutch size, female body

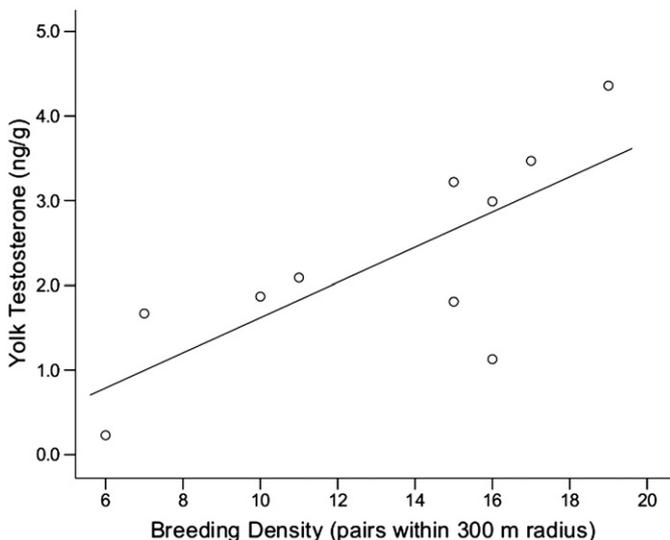


Fig. 1. Regression of average tree swallow breeding density and concentration of yolk testosterone from eggs collected in 2009 when nest boxes were randomly placed.

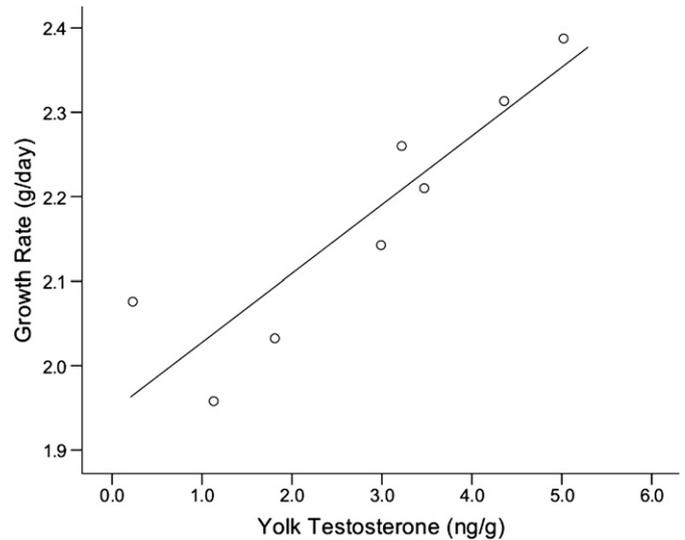


Fig. 2. Regression of yolk testosterone concentrations and average brood growth rate in tree swallows in 2009 when nest boxes were randomly placed.

condition, pair aggression, feeding rate, yolk corticosterone, or % yolk ($p > 0.14$). However, yolk testosterone was significantly higher at the high-density (2.38 ± 0.22 ng/g) than the low-density site (1.55 ± 0.20 ng/g; overall model: $F_{2,16} = 4.69$, $p = 0.03$; Table 1).

Nestling growth and behavior

The average begging rate, begging posture, % of begs in ideal positions, and breathing rate (raw data) are shown in Table 2 based on the pre- and post-natal site of nestlings. The Repeated Measures General Linear Mixed Model showed that nestlings with a high-density pre-natal site exhibited greater mass gain during the nestling stage, regardless of post-natal site (Table 3; Fig. 3). Additionally, yolk corticosterone was negatively related to mass (Table 3). Yolk testosterone ($\chi^2 = -142.73$, $p < 0.001$), yolk corticosterone ($\chi^2 = -5.25$, $p = 0.02$), begging frequency ($\chi^2 = -4.66$, $p = 0.03$), and % yolk ($\chi^2 = -16.68$, $p < 0.001$) all significantly improved the model fit. Both pre- and post-natal nest were included as random factors, because post-natal nest ($Z = 1.69$, $p = 0.09$) and pre-natal nest ($Z = 1.80$, $p = 0.07$) were important contributors to the model and significantly improved the model fit ($\chi^2 = -66.54$, $df = 2$, $p < 0.001$).

Breathing rate during handling stress was highly repeatable with an intraclass correlation coefficient of 0.80. A *t*-test demonstrated that breathing rate was significantly higher in the first 15 s (41.78 ± 0.95 breaths) than the last 15 s (37.29 ± 0.86 breaths) of a 1 min observation ($t_{2,136} = 3.50$, $p = 0.001$), indicating that breathing rapidly increased in response to handling and began to decrease thereafter.

Table 1

ANCOVA analyses of the effect of site density and inherent aggression levels (as measured with a simulated territorial intrusion) on the total number of natural aggressive interactions and yolk testosterone in adult tree swallows in 2011 using high- and low-density field designs.

	B (SE)	F	p-value
Aggressive interactions			
Site density ^a	2.39 (0.86)	7.74	0.02
Pair aggression	0.27 (0.40)	0.46	0.51
Yolk testosterone (ng/g)			
Site density ^a	0.83 (0.30)	7.85	0.01
Female aggression	0.19 (0.13)	2.12	0.17

^a Estimates are relative to low density.

Table 2

Average begging rate (number of begs per feeding bout), posture while begging (1 = gaping, 2 = gaping while facing up, 3 = gaping and extending neck, and 4 = gaping and lifting body up), percent begs in ideal spots (center and near the box entrance were fed at most often and considered ideal), and breathing rate (breaths per min). Means are followed by standard error.

Pre-natal density	Post-natal density	Begging rate (begs/feed)	Posture	Ideal position (% begs)	Breathing rate (breaths/min)
High	High	2.07 ± 0.34	3.32 ± 0.06	56.75 ± 8.39	162.00 ± 6.57
High	Low	2.21 ± 0.32	3.27 ± 0.07	71.54 ± 6.15	171.00 ± 7.11
Low	High	1.97 ± 0.34	3.13 ± 0.07	55.37 ± 8.10	149.47 ± 4.94
Low	Low	1.88 ± 0.28	3.06 ± 0.09	49.88 ± 8.76	148.82 ± 5.09

Mixed Model analysis showed that breathing rate response was significantly higher in nestlings with a high-density pre-natal site (0.46 ± 0.19) than low-density site (−0.32 ± 0.18; Table 4). Only yolk testosterone ($\chi^2 = -28.99, p < 0.001$) significantly improved the model. Random effects were removed because pre-natal nest explained no variance and post-natal nest ($Z = 0.37, p = 0.72$) was not significant and did not significantly improve the model fit ($\chi^2 = -0.15, df = 1, p = 0.70$).

Mixed Model analysis also showed that competitive ability was significantly higher in nestlings with a high-density pre-natal site (0.32 ± 0.23) compared to those with a low-density pre-natal site (−0.21 ± 0.22; Table 4). Yolk testosterone ($\chi^2 = -31.94, p < 0.001$) and nestling mass ($\chi^2 = -10.65, p = 0.001$) significantly improved the model fit. Only post-natal nest ($Z = 2.02, p = 0.04$) was included as a random effect, because pre-natal nest was not significant ($Z = 0.32, p = 0.75$). Including post-natal nest significantly improved the model fit ($\chi^2 = -10.73, df = 1, p = 0.001$), while pre-natal nest did not ($\chi^2 = -0.12, df = 1, p = 0.73$). In addition, competitive ability significantly influenced the proportion of feeds nestlings received. Nestlings with higher competitive ability scores received a significantly higher proportion of the food ($R^2 = 0.09, F_{1,67} = 6.19, p = 0.015$). Competitive ability, however, explained only a small amount of variance, most likely because siblings can restrict the proportion of feeds others will receive so it relies on the nest environment. Using a Paired *t*-test between high and low competitive siblings within a nest, we found that siblings with higher-competitive scores received a greater proportion of the food (32.41% ± 2.55) than their less-competitive siblings (20.42% ± 3.11; $t_{2,16} = 2.30, p = 0.04$).

Table 3

Repeated Measures Linear Mixed Model analysis testing the effect of breeding density on nestling growth in tree swallows in 2011 using high- and low-density field designs. The test statistic for variables in the model is *F* and it is χ^2 for factors excluded from the model. For the variables excluded, none make the model significantly better (more negative χ^2) when added using forward selection procedures.

	Denominator df	Estimate (SE)	<i>F</i> / χ^2	<i>p</i> -value
Variables included				
Pre-natal site ^a	185.7	0.36 (0.16)	5.28	0.02
Post-natal site ^a	170.0	−0.18 (0.15)	1.31	0.25
Begging rate	36.6	0.03 (0.02)	3.74	0.06
Yolk testosterone (ng/g)	9.3	0.11 (0.15)	0.50	0.50
Yolk corticosterone (ng/g)	18.1	−0.57 (0.23)	5.85	0.03
% Yolk	33.9	−0.01 (0.02)	0.28	0.60
Nestling age ^b	57.5	d5 5.07 (0.15)	3128.79	<0.001
		d8 13.17 (0.24)		
		d11 18.42 (0.31)		
		d14 18.79 (0.26)		
Variables excluded				
Brood size			−0.24	0.62
Hatch date			−0.24	0.62
Feeding rate			−0.08	0.77
Begging success			0.00	1.00

Pre- and post-natal nest as random effects.

^a Estimates are relative to low density.

^b Estimates are relative to d2 mass.

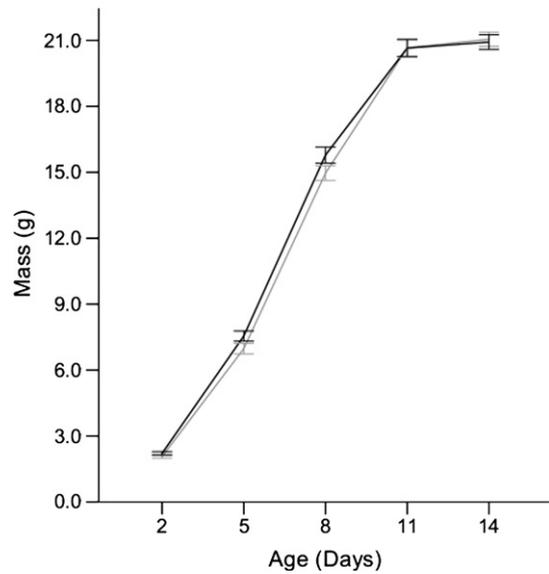


Fig. 3. Effect of pre-natal site density on growth prior to fledging in nestling tree swallows in 2011 using high- and low-density field designs; high-density pre-natal sites are represented by the black line and low-density pre-natal sites are represented by the gray line. Bars represent ± 1 standard error.

Discussion

Correlational study

In 2009, tree swallows settled randomly placed nest boxes such that there was variation in breeding density. Females breeding in high densities laid eggs with greater yolk testosterone concentrations and broods exposed to higher yolk testosterone concentrations grew significantly faster. These data suggest that density can create natural variation in yolk testosterone and, as a consequence, alter offspring growth.

Table 4

Linear Mixed Model analysis testing the effect of breeding density on breathing rate response to a stressor and competitive ability in nestling tree swallows in 2011 using high- and low-density field designs. The test statistic for variables in the model is *F* and χ^2 for factors excluded from the model. For the variables excluded, none make the model significantly better (more negative χ^2) when added using forward selection procedures.

	Denominator df	Estimate (SE)	<i>F</i> / χ^2	<i>p</i> -value
Breathing rate response				
Variables included				
Pre-natal site ^a	53	0.78 (0.28)	7.65	0.01
Post-natal site ^a	53	−0.06 (0.23)	0.07	0.80
Yolk testosterone (ng/g)	53	−0.32 (0.19)	2.86	0.10
Variables excluded				
Mass at fledging (g)			−2.81	0.09
Brood size			−0.44	0.51
Hatch date			−0.41	0.52
% Yolk			−0.02	0.89
Yolk corticosterone (ng/g)			−0.01	0.92
Competitive ability				
Variables included				
Pre-natal site ^a	39.6	0.52 (0.25)	4.47	0.04
Post-natal site ^a	15.2	0.01 (0.37)	0.001	0.97
Yolk testosterone (ng/g)	41.5	0.14 (0.17)	0.68	0.41
Mass at fledging (g)	50.8	−0.02 (0.07)	0.14	0.71
Variables excluded				
Yolk corticosterone (ng/g)			−1.33	0.25
Hatch date			−0.82	0.37
% Yolk			−0.64	0.42
Brood size			−0.57	0.45
Feeding rate			−0.04	0.84

Post-natal nest as random effect in the competitive ability model.

^a Estimates are relative to low density.

Thus, in 2011, we placed nest boxes to create high- and low-density sites to encourage greater variation in breeding density.

Density manipulation

The nest box manipulation in 2011 created breeding sites that caused significant differences in settlement and aggression of tree swallows. Females nesting in the high-density site experienced significantly higher breeding densities than females nesting in the low-density site. Moreover, females experienced a greater number of aggressive interactions at the high- than low-density site and these data corroborate those of Male et al. (2006). Brown et al. (2005) found in the closely related cliff swallow (*Petrochelidon pyrrhonota*) that testosterone levels, which could be indicative of aggressive phenotypes, influence the colony size individuals choose to breed. However, we found that inherent aggression levels did not differ between the sites. This suggests that the phenomenon seen in the high-density site are due to the density manipulation and are not a product of inherently more aggressive birds choosing to settle high-density sites.

No reproductive parameters differed between the sites, except that females in high-density laid eggs with significantly higher yolk testosterone concentrations than those in low-density. Several studies have found that high densities increase yolk testosterone (Hargitai et al., 2009; Mazuc et al., 2003; Pilz and Smith, 2004; Reed and Vleck, 2001; Whittingham and Schwabl, 2001). This relationship is commonly attributed to increased aggression and testosterone in females nesting in high densities; however, we did not measure female testosterone levels. Circulating testosterone is dynamic during follicle development and does not consistently correlate with that found in the yolk. To account for the relationship between social interactions, female testosterone, and yolk testosterone, Muller et al. (2011) suggested that females produce gonadotropin-releasing hormone when challenged which simultaneously causes a transient increase in circulating testosterone and increases testosterone production in ovarian follicles. Thus, a social challenge may increase both yolk and circulating testosterone, but yolk testosterone is not necessarily derived from circulating levels (Muller et al., 2011). Therefore, measuring female testosterone levels would have contributed little to this study given that our data are able to show that greater breeding densities increase number of social challenges and yolk testosterone concentrations.

Nestling growth and behaviors

Breeding density significantly influenced offspring phenotype such that nestlings hatched at high-density grew faster, were more competitive, and had higher breathing rate responses regardless of post-natal density. Due to the partial cross-foster, these phenotypic differences are solely a result of exposure to high-density breeding environments during pre-natal development. The one factor that significantly differed between high- and low-density breeding sites was yolk testosterone, making it the likely source of variation observed in offspring phenotype.

Nestlings hatched from the high-density site showed faster growth and a greater competitive ability than those from low-density. Numerous studies have found that artificially increased yolk testosterone can elicit faster growth (Muller et al., 2007; Navara et al., 2005, 2006a; Pilz et al., 2004; Schwabl, 1996) and more aggressive and competitive behaviors (Eising et al., 2006; Muller et al., 2009; von Engelhardt et al., 2006); however, our study shows this in response to a naturally-derived maternal effect. One possible explanation for these results could be that yolk testosterone increased metabolic rates (Nilsson et al., 2011; Tobler et al., 2007) creating a higher food demand and increased begging vigor. Accordingly, studies have found that high yolk testosterone causes enhanced begging (Eising and Groothuis, 2003; Schwabl, 1993; von Engelhardt et al., 2006) and our data show a positive trend between begging rate and nestling growth. Our data also show that more competitive nestlings obtained a larger percentage of

the food, which is in agreement with other studies (Gottlander, 1987; Kolliker and Richner, 2004; McRae et al., 1993). Exposure to elevated yolk testosterone can facilitate competitiveness, such as enhanced begging posture, by positively affecting development of neck muscles (Lipar and Ketterson, 2000). Therefore, enhanced competitive ability may be the mechanism responsible for increased growth in nestlings hatched from eggs with high yolk testosterone. This may also explain why yolk corticosterone, which decreases begging rates (Rubolini et al., 2005; Wada and Breuner, 2008), had a negative effect on nestling growth, a result consistent with other studies (Hayward and Wingfield, 2004; Saino et al., 2005; Wada and Breuner, 2008).

Breathing rate during handling stress was significantly higher in nestlings from high- compared to low-density pre-natal sites. Several studies have demonstrated that a higher breathing rate response to a stressor is associated with an active coping style (Bell et al., 2010; Boon et al., 2007; Fucikova et al., 2009; van den Brink et al., 2012; but see Carere and van Oers, 2004; David et al., 2012). Specifically, nestling birds with higher breathing rates during handling stress are more exploratory and have higher activity levels (Fucikova et al., 2009; van den Brink et al., 2012). We were unable to measure these traits in altricial nestlings because their behaviors are ill-defined, so we cannot conclusively imply that nestlings with higher breathing rate responses have active coping styles. However, there are several potential mechanisms for our findings that support the link between higher breathing rates and active coping styles. We found that breathing rate was significantly higher in the first seconds of being handled; possibly suggesting this was a sympathetic response and, thus, a component of an active coping style (Chichinadze and Chichinadze, 2008; Koolhaas et al., 1999, 2010). Unfortunately, there are no studies that clearly link breathing rate and sympathetic response. Breathing rate and heart rate are, however, closely related (Ellis et al., 2006) and there is evidence from avian species that heart rate rapidly increases as a sympathetic response to acute restraint stress (Cabanac and Guillemette, 2001; Cyr et al., 2009; Korte et al., 1999). An alternative explanation is that differences in metabolic rates may have caused differences in breathing rate. Breathing rates in nestlings from high-density may always be relatively higher than nestlings hatched from low-density. Indeed, yolk testosterone increases metabolic rates (Nilsson et al., 2011; Tobler et al., 2007) and higher breathing rates are associated with higher metabolic rates (Arens and Cooper, 2005; Furilla and Bernstein, 1995; Pilarski and Hempleman, 2007; Vleck and Bucher, 1998). A high metabolic rate is also related to active coping styles because active behaviors (i.e., greater activity, boldness, and aggression) are energetically costly (Careau et al., 2008). While we can only suggest possible mechanisms to explain our findings, our data show clear differences in the breathing rate response of nestlings from high- versus low-density pre-natal sites that may ultimately be indicative of coping style. Our study is also one of the first to show that pre-natal exposure to higher breeding densities leads to greater breathing rates during handling stress.

Implications for selection

It is intriguing to postulate that increased yolk testosterone concentrations in response to high-density environments are an adaptation to facilitate either increased reproductive success in females or a competitive advantage for nestlings. It is highly probable that selection has occurred at the maternal level (Marshall and Uller, 2007), because this maternal effect is driven by the response of a female to her breeding environment. Only the most aggressive females obtain a breeding cavity in a high-competition environment creating positive selection for this trait (Rosvall, 2008). High yolk testosterone levels could be a physiological by-product of the aggression needed to obtain and maintain a breeding site. Thus, selection for the most aggressive females would act to propagate this maternal effect at high densities. Furthermore, if high yolk testosterone levels create nestlings with a phenotype better suited for

high-density environments, females are further increasing their reproductive success leading to stronger selection.

It is unclear what adaptive purpose this maternal effect serves nestlings, whether it could be beneficial post-fledging in a high-density environment or if nestlings are more likely to breed in a site similar to that of their mother. High-density environments decrease fledgling success (McCleery and Perrins, 1985); therefore, any advantage such as faster growth, greater competitive ability, or more active coping mechanisms would be beneficial in the fledgling stage. Alternatively, cliff swallows have demonstrated a heritable component to their breeding density choice (Brown and Brown, 2000), suggesting that tree swallows may also return to a breeding density similar to that of their mother. If this is the case, high yolk testosterone makes more competitive offspring which would then be more likely to acquire nesting cavities when breeding in similar high-density environments (Rosvall, 2008). Nestling survival would need to be reassessed either prior to migration or in their first breeding year, at which time reproductive success could be determined to definitively determine how selection acts on offspring.

Conclusions

It is generally thought that maternal effects are shaped by natural selection to act as mechanisms for adaptive phenotypic response to environmental heterogeneity. Maternal effects could be an adaptive means by which a female can maximize reproductive success and change the phenotype of her offspring hormonally in preparation for a given environment (Carere and Balthazart, 2007; Groothuis et al., 2005; Mousseau and Fox, 1998). This study shows that high-density environments elicit more social aggression and consequently more yolk testosterone, the nestlings from which grow more quickly and have a greater competitive ability and breathing rate response to a stressor. This study is one of the first to provide an environmental context for this maternal effect and demonstrate that maternally-derived yolk testosterone creates phenotypes well-suited for competitive environments, suggesting they are potentially adaptive.

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