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Quantification of 27 yolk steroid hormones in seven shrubland bird species: interspecific patterns of hormone deposition, and links to life history, development, and predation risk

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Abstract:

Steroid hormones play critical organizational and activational roles during vertebrate development, impacting everything from sexual differentiation to metabolic activity. For oviparous species such as birds, these hormones are transferred from female to egg during follicle maturation, and differences in relative and absolute concentrations of the steroid hormones may reflect differences in life history, developmental, and ecological conditions. Prior work on yolk steroid hormones has focused on a handful of candidate hormones (e.g. testosterone, androstenedione, and corticosterone), but we used high-performance liquid chromatography with tandem mass spectroscopy (LC-MS-MS) to quantify 27 yolk steroids from the eggs of seven shrubland bird species. In addition to comparing steroid profiles across species, we conducted exploratory analyses to determine how the hormones clustered using a principal component (PC) approach, and if PCs were correlated with aspects of egg resources (relative egg size, proportion yolk), life-history traits (embryonic and nestling development speed), and nest-predation risk (daily-survival-rate “DSR”). We documented substantial interspecific variation in both absolute and proportional endocrine profiles. PCAs indicated that glucocorticoids generally clustered together (PC1), but other classes of steroids did not. PCs 2 and 3 strongly covaried with egg resources, DSR and development speed, suggesting they reflect adaptive patterns of maternal hormone deposition.

Keywords: American robin; avian community; brown-headed cowbird; brown thrasher; *Cardinalis cardinalis*; *Dumetella carolinensis*; eastern towhee; egg composition; field sparrow; gray catbird; life history; maternal effects; *Molothrus ater*; nest predation; northern cardinal; *Pipilo erythrophthalmus*; shrubland; *Spizella pusilla*; steroid hormones; *Toxostoma rufum*; *Turdus migratorius*; yolk

Introduction:

Female birds have the ability to impact the condition and survival of their offspring via differential allocation of egg resources. These maternal effects can reflect condition-dependent allocation strategies, in which the female's condition shapes patterns of allocation, or they can occur as the result of adaptive allocation, in which the female adjusts resources based on the perceived value of the offspring and prevailing conditions. At the species-level, patterns of egg resource allocation should reflect interspecific differences in life history, development speed, and predation risk. The deposition of yolk steroid hormones in particular is important because these hormones play critical organizational and activational roles during vertebrate development, influencing a wide range of processes from sexual differentiation to metabolic activity (reviewed in Groothuis et al. 2005). Due to logistical constraints, however, only a small fraction of the steroid hormones that a female deposits into the yolk have been studied in depth, especially in wild birds. In this study, we use high-performance liquid chromatography with tandem mass spectroscopy (LC-MS-MS) to quantify 27 yolk steroid hormones to expand our understanding of what steroid hormones are present in the yolks of different species, how absolute and relative concentrations vary by species, and whether hormone groupings are linked to interspecific patterns of life history, development, or nest-predation risk.

In birds, steroid hormones are transferred from the mother to the yolk during the period of follicle maturation (Groothuis et al. 2005). Yolk hormone levels are thought to vary either as a result of passive transfer, in which the levels in the yolk are proportional to circulating levels in the female, or due to active allocation by the female in a putatively adaptive manner (Groothuis and Schwabl 2008). The ability of females to adjust egg hormone levels has attracted considerable attention following Schwabl's work documenting intra-clutch variation in yolk testosterone levels (Schwabl 1993), and there is evidence that females can manipulate different hormones to match current or predicted environmental and ecological conditions, including resource availability (Ruuskanen et al. 2016), and predation risk (Coslovsky et al. 2012). Prior work on yolk steroid hormones has focused on a handful of hormones, including testosterone (T), androstenedione (A4), estradiol (E2), and corticosterone (CORT), but these represent a subset of hormones and metabolites that females transfer to their eggs. There is good evidence that these hormones are important for development, although the role of each hormone in the early stages of development is not well elucidated for most birds.

Sex steroids (androgens and estrogens) have well-documented effects on sexual differentiation (Arnold 2002; Groothuis et al. 2005), but they can also influence non sex-related aspects of development. One of the more robust patterns is that yolk androgens often positively covary with development speed and begging intensity. In high predation-risk areas, however, elevated begging can be detrimental by attracting the attention of nest predators, and females may allocate lower levels of yolk androgens in those areas (Coslovsky et al. 2012; Merrill et al. In Revision). Alternatively, species under high nest-predation risk often exhibit more rapid rates of development (i.e. shorter incubation period and faster time to fledge), and Schwabl et al. (2007) found that species with higher nest-predation rates had elevated yolk T and 5 α -

dihydrotestosterone (5α -DHT), and also that yolk T and 5α -DHT positively covaried with rates of embryonic development, but less so with chick development.

Less work has focused on the impact of yolk estrogens on development, but there is some evidence indicating that yolk estrogens can influence development and survival (Adkins-Regan et al. 1995; von Engelhardt et al. 2004). Yolk glucocorticoids are thought to reflect circulating levels in the female at the time of yolk formation (Hayward and Wingfield 2004) and at high levels may hinder nestling development (Love et al. 2005; Saino et al. 2005; Schoech et al. 2011). There is some indication, however, that exposure to glucocorticoids can positively impact development (e.g. flight efficiency, Chin et al. 2009), although the timing of CORT exposure is presumably important in determining the outcome. Finally, yolk progestogens have been inversely linked to growth rates (Renden and Benoff 1980; Poisbleau et al. 2011) and predation risk (Merrill et al. In Revision), but their role in early development remains unclear.

In addition to differences in yolk steroid hormones, egg size and composition vary dramatically across bird species, and often reflect different life-history strategies (Ricklefs 1977; Sotherland and Rahn 1987). Highly precocial species tend to have relatively large eggs, and a greater proportion of yolk to albumen. For example, Kiwis lay the largest eggs relative to their body size, and 70% of the egg is yolk (Sotherland and Rahn 1987). Kiwis are fully feathered and capable of feeding themselves upon hatching. Altricial species such as Passerines, however, have relatively small eggs with a low percentage (approx. 20%) of yolk (Ricklefs 1977), and the young are featherless and entirely dependent on the parents upon hatching. Within a species, relatively large eggs are thought to be advantageous due to greater total resources (Williams 1994), and egg size is often positively correlated with offspring growth and survival (reviewed in Krist 2011). At a finer resolution, increases in yolk reflect increased lipid content (Williams

1994), and yolk provides 60-90% of the embryo's total energy requirement (Freeman and Vince 1974; Ojanen 1983). Albumen content reflects water and protein availability, which can influence structural growth (Freeman and Vince 1974), although in altricial species, the yolk may also contain approximately 30% of the total egg protein (Carey et al. 1980).

We quantified yolk steroids from the eggs of seven species of North American shrubland birds, and in addition to describing and quantifying hormone profiles across species, we ran principal component analyses (PCA) to examine how the hormones clustered, and if these associations generally mirrored traditional groupings (e.g. androgens grouping together). We also ran exploratory analyses between the top PCs and life history, development, and nest-predation risk parameters. In general, we predicted that hormones would generally cluster by class (e.g. androgen) via the PCA. We also predicted that species with longer pre-hatch and post-hatch development times (i.e. days to hatch and days to fledge, respectively) would allocate relatively lower levels of yolk androgens (*sensu* Schwabl et al. 2007), and higher levels of yolk progestogens (Renden and Benoff 1980; Poisbleau et al. 2011) (See Figure 1 for graphical representation of predictions). If yolk androgens impact survival via begging activity, higher yolk androgens could negatively impact nest daily survival rates (DSR). Alternatively, species with high DSR may escape this pressure, allowing females to deposit higher androgen levels into the eggs, although slower rates of development associated with higher DSR could negate this effect (Bosque and Bosque 1995; Martin 1995; Chiavacci et al. 2015), resulting in reduced yolk androgens and elevated yolk progestogens (Renden and Benoff 1980; Schwabl et al. 2007; Poisbleau et al. 2011). In addition, we predicted that glucocorticoids would positively covary with egg energy investment because females investing at relatively high levels would be energetically taxed themselves and exhibit elevated levels of circulating glucocorticoids (Bonier

et al. 2009). If glucocorticoids positively impact wing development and flight capabilities, there should be an inverse association between yolk glucocorticoids and days to fledge. We tentatively predicted that yolk estrogens would be positively associated with growth and development speed (Adkins-Regan et al. 1995; von Engelhardt et al. 2004), but negatively associated with DSR if nest-predation risk influenced patterns of growth.

2 Methods:

Study Species:

We focused on six species of open-cup nesting shrubland birds that are abundant in the Midwest US, and one brood parasite (Table 1). These species breed in approximately the same habitats, but differ from each other in a number of life-history parameters, incubation duration, days to fledge, proportion of female mass each egg comprises, proportion of each egg the yolk comprises, as well as nest-predation risk (Table 1).

Nest Searching and Egg Collection:

We searched for nests from April through July, 2014 at eight shrubland sites in East-central Illinois. Shrubland sites were selected as part of a larger study on nest predation of shrubland birds in Illinois (Chiavacci et al. 2018). East-central sites were within 38 km of 40.180 latitude - 87.968 and longitude. We used systematic searches of shrubland habitats as well as parental behaviors to locate nests. Once found, nests were monitored every three days to determine nest stage and fate (fledge/fail). For the egg composition work we randomly selected one egg per nest, with the exception of one field sparrow nest that was going to be destroyed (all four eggs collected), and we removed one host egg and one cowbird egg from brood-parasitized host nests

that contained at least three host eggs (total eggs collected: 91). We assessed yolk hormone concentrations (see below), as well as absolute and relative amount of yolk and albumen (see below). We candled eggs in the field to assess whether development had occurred, and we collected eggs only if the lack of development could be verified by candling, and/or if we knew that incubation had not yet begun. We wrapped collected eggs in aluminum foil, placed them inside a plastic bag, and froze them at -20°C until composition assessment. For our species-level measures of mean egg size, female mass, and nest-predation risk (see below), we used data collected at the same sites from 2011-2015 as part of related work. We measured egg length and width to the nearest 0.01 mm using digital calipers, and estimated volume using $[0.51 \times (\text{length} \times \text{width})^2]$ (Hoyt 1979). All methods were approved by the University of Illinois Animal Care and Use Committee (protocol # 14072).

Daily Survival Rates:

We calculated nest daily survival rates (DSR) for each species (excepting brown-headed cowbirds) across all sites using the logistic-exposure method (Shaffer 2004). These estimates were based on nest-check data collected over a 5-year period as part of related studies (Chiavacci et al. 2018). The vast majority (95%) of nest failures at our study sites were attributable to nest predation (Chiavacci et al. 2018), therefore DSR should represent an accurate measure of the level of nest-predation risk for each species, with lower DSR representing higher risk conditions.

Egg Composition:

We removed eggs from the freezer, massed them, and then allowed them to thaw at room temperature. During the thawing period, we removed the shell from the still-frozen yolk and albumen. We then weighed the shell, as well as the combined yolk and albumen (± 0.001 g). After the albumen had partially thawed, we extracted the yolk, and weighed the yolk and albumen separately.

Life-history and Development:

We calculated average female mass based on capture data from a related study (Merrill pers. obs.), with the exception of eastern towhees and brown-headed cowbirds for which we captured too few individuals. For these species we used published accounts from studies conducted in the Midwestern United States, and at similar latitudes when possible (see Table 1 for references). We used incubation duration and days to fledge from published accounts (Table 1).

Yolk Hormone Assessment:

To prepare yolk samples (0.5 g) for the quantification of steroids using LC-MS-MS (De Baere et al. 2015), steroids were extracted from yolks using methanol (Kozłowski et al. 2009) by adding 2 ml of 100% methanol and vortexing for 60 sec. Neutral lipids were removed by storing samples at -20°C overnight and then spinning at 2000 rpm at 0°C for 20 min. The supernatant was poured to a 50 ml conical vial and a fresh 2 ml of methanol was added to the remaining pellet and to repeat the extraction process. The resulting 4 ml of methanol was diluted with 46 ml of MilliQ water (Millipore, Bedford, MA, USA) to bring the final volume up to 50 ml and this was subsequently subjected to solid phase extraction of the steroids (Newman et al. 2008). Six

additional yolk samples were spiked with 50 ng of each steroid standard to estimate percent recovery. Steroid standards were purchased from Steraloids, Inc. (Newport, RI, USA) and prepared in methanol at a concentration of 1 mg/ml.

Solid phase extraction was carried out with C18 Sep-pak cartridges (Waters, Ltd., Watford, UK) charged with 5 ml methanol and rinsed with 5 ml of water before the sample was loaded. Samples were run through cartridges under vacuum pressure at a drip rate of approximately 2 ml/min. Cartridges were rinsed with 5 ml of water once the sample had run through. Steroids were finally eluted with 5 ml of diethyl ether and dried under nitrogen gas. Dried samples were then submitted to the Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign for the quantification.

Overall, 27 different steroids were quantified across the eggs of all seven species in a single run. Steroids were quantified via LC-MS-MS with a 5500 QTRAP LC-MS-MS system (AB Sciex, Foster City, CA). The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on a Phenomenex C6 Phenyl column (2.0 × 100mm, 3µm.) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.25 ml/min. The linear gradient was as follows: 0-1 min, 80%A; 10 min, 65%A; 15 min, 50%A; 20 min, 40%A; 25 min, 30%A; 30 min, 20%A; 30.5-38 min, 80%A. The autosampler was set at 5°C and the injection volume was 5 µl. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage of 5500 V. The source temperature was 500 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 36 psi, 50 psi, and 65 psi, respectively. Multiple reaction monitoring (MRM) was used to measure steroids (Supplementary material Table S1)¹ and D9-progesterone (m/z 324.1 → m/z 100.1) was used as an internal standard. For

each steroid, standard curves ranging from 2-1000 pg were used for quantification. To calculate percent recovery in intra-assay variation, six yolk samples were spiked with a standard solution that contained 1000 pg of each steroid.

Statistical Analyses:

We assessed interspecific variation in the concentrations of individual hormones and hormone classes using multivariate analysis of variance (SAS PROC GLM). To account for the correlations among hormones, we sought to reduce the dimensionality of the data using principal components analysis on the correlation matrix of variables (SAS PROC FACTOR). We present loadings for all components with eigenvalues >1 , and interpreted those variables with loadings of $\geq|0.5|$ as characterizing each principal component (PC). We examined differences in total hormone concentration and PC scores among species, and relationships between PC scores and DSR, incubation and nestling period lengths, and relative egg investment using general linear mixed models (SAS PROC MIXED), treating nest identity as a random effect to account for the potential non-independence of cases where more than one egg was collected from a nest.

Results:

Interspecific Patterns of Yolk Hormone Deposition:

There were differences among the species in mean hormone concentration for each hormone category (e.g. androgens; Pillai's Trace = 1.31, $F_{24,320} = 6.51$, $P < 0.0001$), as well as for total hormone concentrations ($F_{6,45} = 13.98$, $P < 0.0001$; Figure 2 E). Progestogens were the dominant

hormone category for all species (Supplementary material Fig. S1)¹, although there was a bimodal distribution in which three of the species (brown-headed cowbird, field sparrow, and northern cardinal) had levels that were nearly an order of magnitude lower than those of the other four species (Figure 2 C). Within each hormone category there were large-scale differences among species in the proportion that each specific hormone and metabolite comprised (Supplementary material Fig. S2)¹. In general, cowbirds had the lowest concentrations for all hormones except androgens (second lowest after brown thrashers) and eastern towhees had the highest (excepting progestogens) (Figure 2). There were also differences among the species in the proportions that each category comprised of the total (Supplementary material Fig. S1)¹. Brown thrashers and gray catbirds had low proportions of androgens, whereas northern cardinals and field sparrows had high proportions of androgens (Supplementary material Fig. S1)¹.

Species differed in concentrations of individual hormones (Pillai's Trace = 3.54, $F_{156,360} = 3.31$, $P < 0.0001$; Table 2). There were differences among the species in the proportion of samples that had detectable levels of each hormone; for example, none of the four catbird eggs had detectable levels of testosterone, whereas all American robin eggs did, while many of the glucocorticoids were present in low/non-detectable concentrations for most of the species (Table 2). Catbirds and towhees had relatively small sample sizes for egg component analyses ($n=4$ for each), but these patterns of hormone presence and absence should be robust to small sample sizes.

Principal Component Analyses:

The first 6 principal components explained 79% of the variation in the concentration of our 27 hormones, with PCs 1–3 explaining 59% of the variation (Table 3). For PC1, 10 of the 11 hormones that loaded most strongly (i.e. had loadings >0.5) were glucocorticoids, and all loaded positively. The remaining hormone was an androgen (11-ketotestosterone) which also loaded positively (Table 3). PCs 2 and 3 each had strong loadings from progestogens, estrogens, and androgens, as well as one glucocorticoid each (Table 3). The remaining 3 PCs had only one or two hormones each that had loadings >0.5 , and are not considered further. Scores for PC1 did not vary among species ($F_{6,63} = 0.74$, $P = 0.621$), whereas PC2 and PC3 scores differed among species ($F_{6,76} = 13.16$, $P < 0.0001$ and $F_{6,56} = 18.10$, $P < 0.0001$, respectively)

Yolk Hormones, Nest Predation, and Development:

Nest daily survival rate was negatively related to PC2, which was characterized by elevated levels of testosterone, DHEA, estradiol, and two forms of progesterone (Table 4, Figure 3A), and positively related to PC3, which was defined by greater concentrations of A4, estrone, and two forms of pregnenolone (Figure 3B). Similarly, PC3 was positively associated with length of both the incubation (Figure 3C) and nestling periods (Figure 3D).

Yolk Hormones and Egg Investment:

PC2 positively covaried with relative egg investment (Table 4, Figure 4A), whereas PC3 negatively covaried with relative egg investment and proportion of yolk (Table 4, Figure 4B and C). There were no other associations between yolk hormones and these parameters (Table 4).

Discussion:

Using a non-antibody based method (LC-MS-MS) for detecting and quantifying yolk steroid hormones (De Baere et al. 2015, Larsen et al. 2015), we assessed 27 different yolk hormones and metabolites within a community of shrubland birds. We examined broad patterns of yolk steroid hormone deposition across species and found substantial interspecific variation in hormone presence and absence, hormone concentrations, the proportion of a hormone category (e.g. androgens) that a given hormone comprised, and the proportion of total steroid hormones that a given hormone category comprised. These differences indicate that while these species are relatively closely related (all in the order Passeriformes) and generally share a common habitat, there are noteworthy endocrinological differences in the early embryonic environment across species. We found preliminary but strong evidence that interspecific variation in hormone profiles was linked to variation in nest daily survival rate (DSR), development speed of eggs and nestlings, and egg investment.

Progestogens occurred at very high levels, and were an order of magnitude more abundant than the other hormones (Table 2, Supplementary material Fig. S1)¹, as has been observed in other passerines (Lipar et al. 1999; Paitz et al. 2011). In particular, progesterone and pregnenolone were present in the highest concentrations, and were documented in every sample. Of interest was a pattern in which three of the species (cowbird, field sparrow, and cardinal) had total progestogen concentrations that were almost an order of magnitude lower than those of the other four species. The cause of this difference is not clear, but it could be related to total breeding season egg production. Cowbirds likely produce the highest number of eggs over the course of a season as a result of their brood-parasitic life-history strategy (Scott and Ankney

1980; Fleischer et al. 1987; Holford and Roby 1993), and field sparrows and cardinals may also have elevated numbers due to increased number of re-nesting attempts (Halkin and Linville 1999; Carey et al. 2008; A. Rodewald pers. comms). In support of this idea, field sparrows and cardinals had two of the three lowest rates of nest survival at our sites (Figure 3), and females that produce numerous eggs over an extended period may face physiological constraints (Ward 1996; Williams 2005; Nager 2006; Merrill et al. 2013). If they are limited in their ability to produce progestogens, or if there are costs associated with having elevated levels in circulation, females of these species may decrease the per-egg rates of progestogen deposition. Conversely, these differences could be a product of species-level variation in yolk steroid hormone synthesis pathways, or hormone receptor sensitivity/density in the developing embryos. The fact that progestogen levels were substantially lower in these three species compared to the other four suggests that this is a life-history difference and not one that is physiologically tied to the number of clutches an individual female produces over the course of the season. We would expect greater variation and more overlap between species if these differences were primarily due to egg sampling being biased towards later nests in species with low DSR.

In addition to the interspecific differences in the concentrations of progestogens, some of the patterns between PCs and DSR, development, and egg resources are likely driven by species-level differences in maternal deposition of progestogens, and the likely downstream consequences via steroid hormone synthesis pathways (Supplementary material Fig. S3)¹. PC2 reflects high progesterone, 17 α -hydroxyprogesterone, an androgen precursor (dehydroepiandrosterone), and active sex steroids (testosterone and estradiol) (Table 3). The same enzyme (17 β -hydroxysteroid dehydrogenase- “17 β HSD”) is responsible for producing testosterone (from androstenedione) and estradiol (from estrone) (Payne and Hales 2004), and it

may be that having high progesterone results in an increased propensity to produce testosterone and estradiol, possibly by upregulating 17 β HSD. The inverse association between PC2 and DSR supports our prediction that birds under elevated risk of nest predation would have higher levels of yolk androgens. Birds exposed to elevated rates of nest predation should be under selection for increased embryonic development, but reduced begging activity if nest predators cue in on nestling vocalizations or activity rates of the adults (e.g. Mullin and Cooper 1998; Haff and Magrath 2011). Androgens can speed up development in two ways: 1) by accelerating rates of embryonic development (Eising et al. 2001; Schwabl et al. 2007), or 2) by increasing rates of post-hatching development (Schwabl 1996). Changes in embryonic development due to elevated levels of yolk androgens likely occur via direct action on somatic tissues (i.e. increased anabolic metabolism (Navara and Mendonca 2008)). Changes in nestling development, on the other hand, have been tied to behavioral changes, such as increases in rates of begging intensity (Eising and Groothuis 2003), or potentially to carryover effects of increased embryonic development (i.e. shorter incubation period, earlier access to food resources; Eising et al. 2001; Schwabl et al. 2007). Schwabl et al. (2007) found that yolk T and 5 α -dihydrotestosterone (DHT) were positively associated with predation risk (at the individual level) and with embryonic development (especially DHT), as well as with chick development, but to a lesser degree. In contrast to that study, PC2 was not associated with either embryonic or nestling development speed in our study.

PC3, however, was strongly associated with DSR, development speed, and egg investment, and may represent an alternate strategy of maternal hormone deposition. PC3 was marked by high levels of pregnenolone, 17 α -hydroxypregnenolone, and sex steroid precursors (estrone and androstenedione) (Table 3), and the association between PC3, DSR, and

development speed were markedly stronger than for PC2 (Table 4). If PC3 most strongly explains the ecological and life-history variation among species, the hormones that load most strongly onto PC3 are likely to be of particular interest to researchers. The variation in androstenedione (A4) and 17α -hydroxypregnenolone may be key components of this hormone investment strategy based on the strong positive associations between A4 and 17α -hydroxypregnenolone and both DSR and incubation duration (Table 4). The overarching pattern is that species with higher PC3 scores had higher DSR, and longer development times (both embryonic and nestling). These findings support our predictions and coincide with prior work demonstrating inverse associations between yolk progestogens and growth rates (Renden and Benoff 1980; Poisbleau et al. 2011), and nest-predation risk (Merrill et al. In Revision). The mechanisms whereby yolk progestogens influence growth rates in the embryo are not well understood, but these data provide additional evidence that they are important mediators of avian embryonic development and should be examined further. Conversion of A4 to testosterone may be a critical regulatory point in the response to nest predation risk.

Both PCs 2 and 3 were associated with relative female egg investment, and the general patterns between the respective PCs and DSR and egg investment (Figures 3 and 4) indicates there may be a trade-off between egg energy investment and deposition of certain yolk hormones. Birds under high predation risk align with the PC2 strategy in which they invest more energy resources into their eggs for rapid development, and have higher levels of progesterone and T (among others), whereas those species under reduced nest-predation risk follow the PC3 strategy, invest relatively lower levels of energy resources, and have higher levels of pregnenolone and A4 (among others). These patterns are purely descriptive at this point, but they do match the predicted patterns, and warrant further investigation.

The majority of the glucocorticoid hormones and metabolites loaded onto PC1, which may reflect activation of the HPA axis in the female. This PC did not vary by species, but exhibits a loosely bimodal distribution, in that a few individuals of each species exhibit high glucocorticoid levels. These individuals likely experienced an activation of the HPA axis during egg production and glucocorticoids produced by the adrenal gland were passively transferred to the egg. Many of the glucocorticoid hormones and metabolites occurred at very low concentrations, and most were present in a small percentage of the total samples. The glucocorticoids present in the highest percent of samples were cortisol, 5 β -dihydropyrene, and deoxycorticosterone (Table 2). There is some evidence that females limit deposition of glucocorticoids to the eggs as a way to minimize the embryo's exposure to them (Vassallo et al. 2014). Glucocorticoids can have strong programming effects on developing organisms (Love and Williams 2008; Zimmer et al. 2013), and in many cases, individuals exposed to elevated glucocorticoids during development express phenotypic traits that should be beneficial under stressful conditions, but perform poorly under normal conditions compared to unstressed phenotypes (*sensu* Monaghan 2008). Concentrations alone do not tell the complete story however, and without knowing receptor levels or sensitivities, we cannot definitively determine if these low concentrations reflect a strategy of limiting exposure to the embryo. They do, however, provide insight into which glucocorticoids may be of most importance to the developing embryo, and it is noteworthy that the glucocorticoids generally used to simulate high levels of maternally transferred glucocorticoids (corticosterone and cortisol) were present in very low concentrations, and undetectable in many individuals (Table 2). It may be that manipulating 5 β -dihydropyrene or deoxycorticosterone would better mimic natural variation in maternal stress hormone deposition.

There has been substantial interest in patterns of maternal investment within and across species, and there is good evidence that females can impact the fitness prospects of their offspring via changes in egg resource deposition. Despite the interest in maternal effects in birds, we still have a limited understanding of how environmental factors such as nest predations risk, and life-history traits such as incubation period, impact patterns of maternal resource allocation (but see e.g. Martin 1995; Conway and Martin 2000; LaManna and Martin 2017; Ton and Martin 2017). This is especially true for maternally-derived yolk hormones such as steroid hormones. To date, most of the studies on yolk steroid hormones have focused on a few hormones such as T, DHEA, A4, and CORT among others. This somewhat narrow scope is mostly a product of logistical constraint in assessing multiple types of hormones from one sample. LC-MS-MS has recently been developed as an alternative approach to quantifying steroids because it can provide more specificity and selectivity than traditional antibody based approaches (De Baere et al. 2015, Larsen et al. 2015). By quantifying 27 steroids simultaneously, we were able to characterize more complete endocrine profiles with egg yolks. We acknowledge that there are other hormones we did not quantify (e.g. 5 α -DHT) which could play a role in early embryonic development, although prior work in European starlings indicated that 5 α -DHT does not appear to do so as a maternally derived yolk hormone (Paitz et al. 2011). In addition, there are some limitations to LC-MS-MS in that extraction techniques cannot be optimized for any one steroid. It is also not always feasible to examine the effect of matrix effects (which could differ for each bird species) on each individual steroid. Additional studies would be necessary to improve extraction efficiencies and precision for specific steroids of interest as has been done with corticosterone and gull eggs (De Baere et al. 2015; Larsen et al. 2015). Despite these limitations, our steroid patterns are still very consistent with patterns reported for other bird species using antibody

based techniques. Similar to previous reports, we found that progesterone, along with other progestogens, were the most abundant of the yolk steroids (Lipar et al. 1999; Paitz et al. 2011). Androgens tended to be the next most abundant class of steroids (Lessells et al. 2016), followed by relatively low levels of estrogens (Lipar et al. 1999; Lessells et al. 2016) and glucocorticoids (De Baere et al. 2015; Lessells et al. 2016).

Based on the strong associations between PCs 2 and 3, and DSR, development speed, and egg investment, the species-level differences in hormone concentrations, hormone proportions, and PC clusterings do not appear to be random or an artifact of phylogeny, but likely reflect adaptive patterns of maternal hormone deposition in response to ecological pressures. We again want to point out the exploratory nature of these analyses, and that some of these parameters are linked. Species with higher DSR, for example, generally have longer developmental periods (incubation and nestling). They are thus not entirely independent of one another, and care should be taken when considering the patterns between PCs and egg/nestling traits. In addition to the PCs, we examined traditional groupings of hormones (e.g. total progestogens), and while we initially found evidence of associations between total progestogens and DSR and incubation length (data not shown), our PC loadings for PCs 2 and 3 underscore the potential pitfalls of grouping hormones together (Table 3).

Overall these data highlight just how complex the early endocrine environment is within avian eggs, and that we are just beginning to understand which, if any, of these compounds are impacting embryonic development. Our data thus provide a foundation upon which future studies can build by more specifically targeting yolk steroid hormones that may be important in the development of avian embryos and nestlings. Although sample sizes are small for some species, and hormone concentrations are low/non-detectable in many cases, we documented

several associations between species-level DSR, patterns of development and yolk steroid hormones in a community of shrubland birds, highlighting a number of important avenues for future research into the functioning of maternal investment strategies and avian embryonic development.

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Literature Cited:

- Adkins-Regan, E., Ottinger, M.A., and Park, J. 1995. Maternal transfer of estradiol to egg yolks alters sexual differentiation of avian offspring. *J. Exp. Zool.* **271**(6): 466–470. doi:10.1002/jez.1402710608.
- Arnold, A. P. 2002. Concepts of genetic and hormonal induction of vertebrate sexual differentiation in the twentieth century, with special reference to the brain. *In* *Hormones, brain and behavior*, vol 4. *Edited by* D.W. Pfaff, A.P. Arnold, A.M. Etgen, S.E. Fahrbach, and R.T. Rubin. Academic Elsevier, Amsterdam, pp. 105–136.
- Barbour, R. 1951. Observations on the breeding habits of the red-eyed towhee. *Am. Midl. Nat.* **45**(3): 672–678. doi:10.2307/2421997.
- Bent, A.C. 1968. Life histories of North American cardinals, grosbeaks, towhees, finches, sparrows, and allies (Part 1). *U.S. Natl. Mus. Bull.* No. 237.
- Best, L.B. 1978. Field Sparrow Reproductive Success and Nesting Ecology. *Auk*, **95**(1): 9–22.
- Bonier, F., Moore, I.T., Martin, P.R., and Robertson, R.J. 2009. The relationship between fitness and baseline glucocorticoids in a passerine bird. *Gen. Comp. Endocrinol.* **163**(1–2): 208–213. doi:10.1016/j.ygcen.2008.12.013.
- Bosque, C., and Bosque, M. 1995. Nest predation as a selective factor in the evolution of developmental rates in altricial birds. *Am. Nat.* **145**(2): 234–260. doi:10.1086/285738.
- Carey, M., Burhans, D.E., and Nelson, D.A. 2008. Field Sparrow (*Spizella pusilla*). *In* *The Birds of North America Ithaca*. *Edited by* P.G. Rodewald. Cornell Lab of Ornithology, DOI: [10.2173/bna.103](https://doi.org/10.2173/bna.103).
- Carey, C., Rahn, H., and Parisi, P. 1980. Calories, water, lipid and yolk in avian eggs. *Condor*, **82**(3): 335–343. doi:10.2307/1367405.

- Cavitt, J. F., and Haas, C.A. 2014. Brown Thrasher (*Toxostoma rufum*). In The Birds of North America Ithaca. Edited by P.G. Rodewald. Cornell Lab of Ornithology, DOI: [10.2173/bna.557](https://doi.org/10.2173/bna.557).
- Chiavacci, S.J., Benson, T.J. and Ward, M.P. 2018. Linking landscape composition to predator-specific nest predation requires examining multiple landscape scales. J. Appl. Ecol. **55**(4): 2082-2092. doi.org/10.1111/1365-2664.13090.
- Chiavacci, S.J., Ward, M.P., and Benson, T.J. 2015. Why fledge early in the day? Examining the role of predation risk in explaining fledging behavior. Behav. Ecol. **26**(2): 593–600. doi:10.1093/beheco/aru236.
- Chin, E.H., Love, O.P., Verspoor, J.J., Williams, T.D., Rowley, K., and Burness, G. 2009. Juveniles exposed to embryonic corticosterone have enhanced flight performance. Proc. R. Soc. Lond. B Biol. Sci. **276**(1656): 499–505. doi:10.1098/rspb.2008.1294.
- Clench, M.H., and Leberman, R.C. 1978. Weights of 151 species of Pennsylvania USA birds analyzed by month age and sex. Bull. Carnegie Mus. Nat. Hist. (5): 1–87.
- Conway, C.J., and Martin, T.E. 2000. Evolution of passerine incubation behavior: Influence of food, temperature, and nest predation. Evolution, **54**(2): 670–685.
- Coslovsky, M., Groothuis, T., de Vries, B., and Richner, H. 2012. Maternal steroids in egg yolk as a pathway to translate predation risk to offspring: Experiments with great tits. Gen. Comp. Endocrinol. **176**(2): 211–214. doi:10.1016/j.ygcen.2012.01.013.
- De Baere, S., Larsen, T.R., Devreese, M., De Backer, P., De Neve, L., Fairhurst, G., Lens, L., and Croubels, S. 2015. Use of LC-MS-MS as an alternative to currently available immunoassay methods to quantitate corticosterone in egg yolk and albumen. Anal. Bioanal. Chem. **407**(15): 4351–4362. doi:10.1007/s00216-014-8269-7.

- Dickinson Jr., J.C. 1968. Rufous-sided Towhees: eastern US subspecies. *In* Life histories of North American cardinals, grosbeaks, buntings, towhees, finches, sparrow and allies. Edited by O.L. Austin. US National Museum Bulletin, 237:562-580.
- Durkin, E.S., Luong, L.T., and Bird, J. 2015. Mechanisms underlying parasite infection: influence of host body mass and age on chewing louse distribution among brown-headed cowbirds. *Parasitol. Res.* **114**(11): 4169–4174. doi:10.1007/s00436-015-4648-z.
- Edington, J., and Edington, M. 1972. Spatial patterns and habitat partition in breeding birds of an upland wood. *J. Anim. Ecol.* **41**(2): 331-357. doi:10.2307/3472.
- Eising, C.M., Eikenaar, C., Schwabl, H., and Groothuis, T.G.G. 2001. Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. *Proc. R. Soc. Lond. B Biol. Sci.* **268**(1469): 839–846. doi:10.1098/rspb.2001.1594.
- Eising, C.M., and Groothuis, T.G.G. 2003. Yolk androgens and begging behaviour in black-headed gull chicks: an experimental field study. *Anim. Behav.* **66**: 1027–1034. doi:10.1006/anbe.2003.2287.
- Fleischer, R., Smyth, A., and Rothstein, S. 1987. Temporal and Age-Related Variation in the Laying Rate of the Parasitic Brown-Headed Cowbird in the Eastern Sierra-Nevada, California. *Can. J. Zool.* **65**(11): 2724–2730.
- Freeman, B.M., and Vince, M.A. 1974. Development of the avian embryo: a behavioural and physiological study. Chapman and Hall. London.
- Groothuis, T.G.G., Muller, W., von Engelhardt, N., Carere, C., and Eising, C. 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neurosci. Biobehav. Rev.* **29**(2): 329–352. doi:10.1016/j.neubiorev.2004.12.002.
- Groothuis, T.G.G., and Schwabl, H. 2008. Hormone-mediated maternal effects in birds:

- mechanisms matter but what do we know of them? *Philos. Trans. R. Soc. Lond. B Biol. Sci. No.* **363**(1497): 1647–1661. doi:10.1098/rstb.2007.0007.
- Haff, T.M., and Magrath, R.D. 2011. Calling at a cost: elevated nestling calling attracts predators to active nests. *Biol. Lett.* **7**(4): 493–495. doi:10.1098/rsbl.2010.1125.
- Halkin, S. L., and Linville, S.U. 1999. Northern Cardinal (*Cardinalis cardinalis* In The Birds of North America Ithaca. *Edited by* P.G. Rodewald. Cornell Lab of Ornithology, DOI: [10.2173/bna.440](https://doi.org/10.2173/bna.440).
- Hayward, L.S., and Wingfield, J.C. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. *Gen. Comp. Endocrinol.* **135**(3): 365–371. doi:10.1016/j.ygcen.2003.11.002.
- Holford, K., and Roby, D. 1993. Factors limiting fecundity of captive brown-headed cowbirds. *Condor* **95**(3): 536–545. doi:10.2307/1369597.
- Howell, J.C. 1942. Notes on the nesting habits of the American Robin (*Turdus migratorius* L.). *Am. Midl. Nat.* **28**: 529–603. doi:10.2307/2420891.
- Hoyt, D. 1979. Practical methods of estimating volume and fresh weight of bird eggs. *Auk*, **96**(1): 73–77.
- Johnson, E. J., and Best, L.B. 1980. Breeding biology of the gray catbird in Iowa. *Iowa State J. Res.* **55**: 171-183.
- Kinser, G.W. 1973. Ecology and behavior of the cardinal *Richmondia cardinalis* (L), in southern Indiana. PhD Thesis. Indiana University, Bloomington.
- Kozlowski, C.P., Bauman, J.E., and Hahn, D.C. 2009. A simplified method for extracting androgens from avian egg yolks. *Zoo Biol.* **28**(2): 137–143. doi:10.1002/zoo.20221.

- Krist, M. 2011. Egg size and offspring quality: a meta-analysis in birds. *Biol. Rev. Camb. Philos. Soc.* **86**(3): 692–716. doi:10.1111/j.1469-185X.2010.00166.x.
- LaManna, J.A., and Martin, T.E. 2017. Seasonal fecundity and costs to lambda are more strongly affected by direct than indirect predation effects across species. *Ecology*, **98**(7): 1829–1838. doi:10.1002/ecy.1860.
- Larsen, T.R., Fairhurst, G.D., De Baere, S., Croubels, S., Muller, W., De Neve, L., and Lens, L. 2015. Novel insights into relationships between egg corticosterone and timing of breeding revealed by LC-MS/MS. *J. Avian Biol.* **46**(6): 643–647. doi:10.1111/jav.00735.
- Lessells, C.M., Ruuskanen, S., and Schwabl, H. 2016. Yolk steroids in great tit *Parus major*. *Behav. Ecol. Sociobiol.* **70**(6): 843–856. doi:10.1007/s00265-016-2107-1.
- Lipar, J.L., Ketterson, E.D., Nolan, V., and Casto, J.M. 1999. Egg yolk layers vary in the concentration of steroid hormones in two avian species. *Gen. Comp. Endocrinol.* **115**(2): 220–227. doi:10.1006/gcen.1999.7296.
- Love, O.P., Chin, E.H., Wynne-Edwards, K.E., and Williams, T.D. 2005. Stress hormones: A link between maternal condition and sex-biased reproductive investment. *Am. Nat.* **166**(6): 751–766. doi:10.1086/497440.
- Love, O.P., and Williams, T.D. 2008. Plasticity in the adrenocortical response of a free-living vertebrate: The role of pre- and post-natal developmental stress. *Horm. Behav.* **54**(4): 496–505. doi:10.1016/j.yhbeh.2008.01.006.
- Martin, T. 1995. Avian life-history evolution in relation to nest sites, nest predation, and food. *Ecol. Monogr.* **65**(1): 101–127. doi:10.2307/2937160.
- Martin, T.E., and Schwabl, H. 2008. Variation in maternal effects and embryonic development rates among passerine species. *Philos. Trans. R. Soc. Lond. B Biol. Sci. No.* **363**(1497):

- 1663–1674. doi:10.1098/rstb.2007.0009.
- Merrill, L., O’Loghlen, A.L., Wingfield, J.C., and Rothstein, S.I. 2013. Immune function in an avian brood parasite and its nonparasitic relative. *Physiol. Biochem. Zool.* **86**(1): 61–72. doi:10.1086/668852.
- Monaghan, P. 2008. Early growth conditions, phenotypic development and environmental change. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* No. **363**(1497): 1635–1645. doi:10.1098/rstb.2007.0011.
- Mullin, S.J., and Cooper, R.J. 1998. The foraging ecology of the gray rat snake (*Elaphe obsoleta spiloides*) - Visual stimuli facilitate location of arboreal prey. *Am. Midl. Nat.* **140**(2): 397–401. doi:10.1674/0003-0031(1998)140[0397:TFEOTG]2.0.CO;2.
- Nager, R.G. 2006. The challenges of making eggs. *Ardea*, **94**(3): 323–346.
- Navara, K.J., and Mendonca, M.T. 2008. Yolk androgens as pleiotropic mediators of physiological processes: A mechanistic review. *Comp. Biochem. Physiol. A* **150**(4): 378–386. doi:10.1016/j.cbpa.2008.05.002.
- Newman, A.E.M., Chin, E.H., Schmidt, K.L., Bond, L., Wynne-Edwards, K.E., and Soma, K.K. 2008. Analysis of steroids in songbird plasma and brain by coupling solid phase extraction to radioimmunoassay. *Gen. Comp. Endocrinol.* **155**(3): 503–510. doi:10.1016/j.ygcen.2007.08.007.
- Nice, M.M. 1953. The question of ten-day incubation periods. *Wilson Bull.* **65**((2)): 81–93.
- Nickell, W. 1965. Habitats territory and nesting of catbird. *Am. Midl. Nat.* **73**(2): 433-. doi:10.2307/2423464.
- Ojanen, M. 1983. Composition of the Eggs of the Great Tit (*Parus major*) and the Pied Flycatcher (*Ficedula hypoleuca*). *Ann. Zool. Fenn.* **20**(1): 57–63.

- Paitz, R.T., Bowden, R.M., and Casto, J.M. 2011. Embryonic modulation of maternal steroids in European starlings (*Sturnus vulgaris*). *Proc. R. Soc. Lond. B Biol. Sci.* **278**(1702): 99–106. doi:10.1098/rspb.2010.0813.
- Payne, A.H., and Hales, D.B. 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr. Rev.* **25**(6): 947–970.
- Poisbleau, M., Demongin, L., Parenteau, C., and Eens, M. 2011. Intra-clutch ratio of yolk progesterone level changes with laying date in rockhopper penguins: A strategy to influence brood reduction? *PLoS One*, **6**(11). doi:10.1371/journal.pone.0027765.
- Renden, J.A., and Benoff, F.H. 1980. Effects of progesterone on developing chick embryos. *Poult. Sci.* **59**(11): 2559–2563.
- Ricklefs, R. 1977. Composition of Eggs of Several Bird Species. *Auk*, **94**(2): 350–356.
- Ricklefs, R.E. 2006. Embryo development and ageing in birds and mammals. *Proc. Biol. Sci.* **273**(1597): 2077–2082. doi:10.1098/rspb.2006.3544.
- Ruuskanen, S., Darras, V.M., de Vries, B., Visser, M.E., and Groothuis, T.G.G. 2016. Experimental manipulation of food availability leads to short-term intra-clutch adjustment in egg mass but not in yolk androgen or thyroid hormones. *J. Avian Biol.* **47**(1): 36–46. doi:10.1111/jav.00728.
- Saino, N., Romano, M., Ferrari, R.P., Martinelli, R., and Moller, A.P. 2005. Stressed mothers lay eggs with high corticosterone levels which produce low-quality offspring. *J. Exp. Zool. Part A* **303A**(11): 998–1006. doi:10.1002/jez.a.224.
- Schoech, S.J., Rensel, M.A., and Heiss, R.S. 2011. Short- and long-term effects of developmental corticosterone exposure on avian physiology, behavioral phenotype, cognition, and fitness: A review. *Curr. Zool.* **57**(4): 514–530.

- Schwabl, H. 1993. Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. U. S. A.* **90**(24): 11446–11450. doi:10.1073/pnas.90.24.11446.
- Schwabl, H. 1996. Maternal testosterone in the avian egg enhances postnatal growth. *Comp. Biochem. Physiol. A* **114**(3): 271–276. doi:10.1016/0300-9629(96)00009-6.
- Schwabl, H., Palacios, M.G., Martin, T.E., Adkins-Regan, A.E.E., and Whitlock, E.M.C. 2007. Selection for rapid embryo development correlates with embryo exposure to maternal androgens among passerine birds. *Am. Nat.* **170**(2): 196–206. doi:10.1086/519397.
- Scott, D.M., and Ankney, C.D. 1980. Fecundity of the Brown-Headed Cowbird in Southern Ontario. *Auk*, **97**(4): 677–683.
- Scott, D.M., and Lemon, R.E. 1996. Differential reproductive success of Brown-headed Cowbirds with Northern Cardinals and three other hosts. *Condor*, **98**(2): 259–271. doi:10.2307/1369144.
- Shaffer, T.L. 2004. A unified approach to analyzing nest success. *Auk*, **121**(2): 526–540. doi:10.1642/0004-8038(2004)121[0526:AUATAN]2.0.CO;2.
- Sotherland, P., and Rahn, H. 1987. On the composition of bird eggs. *Condor*, **89**(1): 48–65. doi:10.2307/1368759.
- Stearns, S.C. 1992. *The Evolution of Life Histories*. First edition. Oxford University Press, Oxford; New York.
- Ton, R., and Martin, T.E. 2017. Proximate effects of temperature versus evolved intrinsic constraints for embryonic development times among temperate and tropical songbirds. *Sci. Rep.* **7**: 895. doi:10.1038/s41598-017-00885-3.
- Vassallo, B.G., Paitz, R.T., Fasanella, V.J., and Haussmann, M.F. 2014. Glucocorticoid metabolism in the in ovo environment modulates exposure to maternal corticosterone in

- Japanese quail embryos (*Coturnix japonica*). Biol. Lett. **10**(11): 20140502.
doi:10.1098/rsbl.2014.0502.
- von Engelhardt, N., Dijkstra, C., Daan, S., and Groothuis, T.G.G. 2004. Effects of 17- β -estradiol treatment of female zebra finches on offspring sex ratio and survival. Horm. Behav. **45**(5): 306–313. doi:10.1016/j.yhbeh.2003.12.009.
- Walkinshaw, L.H., 1968. Eastern field sparrow. In Life histories of North American cardinals, grosbeaks, buntings, towhees, finches, sparrow and allies. Edited by O.L. Austin. US National Museum Bulletin, 237:1217-1235.
- Ward, S. 1996. Energy expenditure of female barn swallows *Hirundo rustica* during egg formation. Physiol. Zool. **69**(4): 930–951.
- Williams, T. 1994. Intraspecific variation in egg size and egg composition in birds - effects on offspring fitness. Biol. Rev. Camb. Philos. Soc. **69**(1): 35–59. doi:10.1111/j.1469-185X.1994.tb01485.x.
- Williams, T.D. 2005. Mechanisms underlying the costs of egg production. BioScience, **55**(1): 39–48. doi:10.1641/0006-3568(2005)055[0039:MUTCOE]2.0.CO;2.
- Woodward, P.W., and Woodward, C.J. 1979. Survival of fledgling brown-headed cowbirds *Molothrus ater*. Bird Banding, **50**(1): 66–68.
- Zimmer, C., Boogert, N.J., and Spencer, K.A. 2013. Developmental programming: Cumulative effects of increased pre-hatching corticosterone levels and post-hatching unpredictable food availability on physiology and behaviour in adulthood. Horm. Behav. **64**(3): 494–500. doi:10.1016/j.yhbeh.2013.07.002.
- Zimmerman, J. L. 1963. A nesting study of the catbird in southern Michigan. Jack-Pine Warbler, **41**:141-160.

Figure Captions:

Figure 1. Predicted associations among nestling daily survival rate, maternal investment, and embryo and nestling development in six shrubland birds (American robin-*Turdus migratorius*, brown thrasher-*Toxostoma rufum*, eastern towhee-*Pipilo erythrophthalmus*, field sparrow-*Spizella pusilla*, gray catbird-*Dumetella carolinensis*, and northern cardinal-*Cardinalis cardinalis*) and a brood parasite (brown-headed cowbird-*Molothrus ater*). Arrows reflect the direction of effect, and dashed arrows signify when there is the potential for bidirectional effects. Plus and minus signs denote whether the effect is positive or negative. Arrows with multiple symbols reflect associations in which there is support for both a positive and negative effect of one parameter on the other. (NA) denotes instances in which there is not expected to be an association, and (?) is for associations in which the directionality is unclear.

Figure 2. Mean $\pm$ SE yolk steroid concentrations for each category (A-androgens, B-Estrogens, C-Progestogens, D-Glucocorticoids) and total summed concentrations (E) for each bird species: American robin (AMRO-*Turdus migratorius*; $n=9$), brown-headed cowbird (BHCO-*Molothrus ater*; $n=22$), brown thrasher (BRTH-*Toxostoma rufum*; $n=11$), eastern towhee (EATO-*Pipilo erythrophthalmus*; $n=4$), field sparrow (FISP-*Spizella pusilla*; $n=23$), gray catbird (GRCA-*Dumetella carolinensis*; $n=4$), and northern cardinal (NOCA-*Cardinalis cardinalis*; $n=14$).

Figure 3. Associations between principal components 2 or 3 from analyses of hormone groupings and nest daily survival rate (A, B), length of the incubation period (C), and length of the nestling period (D) for each of the six nest-building species. Bars represent mean $\pm$ SE. Species examined are: American robin (AMRO-*Turdus migratorius*; $n=9$), brown thrasher (BRTH-*Toxostoma*

rufum; $n=11$), eastern towhee (EATO-*Pipilo erythrophthalmus*; $n=4$), field sparrow (FISP-*Spizella pusilla*; $n=23$), gray catbird (GRCA-*Dumetella carolinensis*; $n=4$), and northern cardinal (NOCA-*Cardinalis cardinalis*; $n=14$).

Figure 4. Associations between principal components 2 or 3 from analyses of hormone groupings and proportion of the female's mass each egg represents (A, B), and the percent of the egg composed of yolk (C) for each of the six nest-building species. Bars represent mean $\pm$ SE. Species examined are: American robin (AMRO-*Turdus migratorius*; $n=9$), brown thrasher (BRTH-*Toxostoma rufum*; $n=11$), eastern towhee (EATO-*Pipilo erythrophthalmus*; $n=4$), field sparrow (FISP-*Spizella pusilla*; $n=23$), gray catbird (GRCA-*Dumetella carolinensis*; $n=4$), and northern cardinal (NOCA-*Cardinalis cardinalis*; $n=14$).

Figure 1

190x107mm (300 x 300 DPI)

Figure 2

Figure 2

279x361mm (300 x 300 DPI)

Figure 3

279x361mm (300 x 300 DPI)

Figure 4

Figure 4

279x361mm (300 x 300 DPI)

Table 1. Summary statistics for maternal investment, development, and daily survival rate data for American robin (AMRO-*Turdus migratorius*), brown-headed cowbird (BHCO-*Molothrus ater*), brown thrasher (BRTH-*Toxostoma rufum*), eastern towhee (EATO-*Pipilo erythrophthalmus*), field sparrow (FISP-*Spizella pusilla*), gray catbird (GRCA-*Dumetella carolinensis*), and northern cardinal (NOCA-*Cardinalis cardinalis*).

Species	Female Mass (g) ^a	Egg Volume (mm ³)	% Female Mass ^b	% Yolk ^c	Incubation Duration (d) ^d	Nestling Period (d) ^e	DSR ^f
AMRO	77.58	6020.05	7.76	24.5	13.0	13.0	0.954
BHCO	35.50*	2929.66	8.25	21.1	11.0	10.5	NA
BRTH	66.04	5093.67	7.71	20.0	12.6	11.5	0.951
EATO	39.30*	3962.82	10.08	20.9	12.0	10.5	0.927
FISP	12.06	1599.44	13.26	28.8	11.0	7.5	0.916
GRCA	35.68	3657.40	10.25	27.8	12.9	10.5	0.964
NOCA	42.00	4333.50	10.32	23.4	12.8	9.5	0.938

- a. Values determined from birds captured at study sites as part of a related study. Asterisks represent birds for which we did not capture enough adult females to calculate mean mass, and for which we used published values from studies conducted in the Midwest at similar latitudes (when possible) (Clench and Leberman 1978; Durkin et al. 2015).
- b. Values are the percent of female mass that the average egg comprises.
- c. Percent of the egg composed of yolk.
- d. Length of the incubation period; values determined based on averages from literature (Howell 1942; Nice 1953; Dickinson 1968; Kinser 1973; Best 1978; Johnson and Best 1980; Cavitt and Haas 2014).

- e. Length of the nestling period; values determined based on average from literature (Howell 1942; Barbour 1951; Zimmerman 1963; Nickell 1965; Bent 1968; Walkinshaw 1968; Best 1978; Woodward and Woodward 1979; Scott and Lemon 1996; Cavitt and Haas 2014).
- f. Nest daily survival rate across all study sites and years (see text for details).

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Table 2. Mean concentrations (ng/g) for each of the 27 steroid hormones quantified in the seven focal bird species, arranged by total concentration across species. *n* represents the total number of eggs used to quantify hormone concentrations for each species, and # represents the number of eggs which had detectable concentrations for each hormone. 0 values are for hormones not detected, or for standard error estimates of hormones detected in one sample. AMRO = American robin, BHCO = brown-headed cowbird, BRTH = brown thrasher, EATO = eastern towhee, FISP = field sparrow, GRCA = gray catbird, and NOCA = northern cardinal.

Steroid Hormones	AMRO <i>n</i> =9			BHCO <i>n</i> =22			BRTH <i>n</i> =11			EATO <i>n</i> =4			FISP <i>n</i> =23			GRCA <i>n</i> =4			NOCA <i>n</i> =14		
	$\bar{X}$	SE	#	$\bar{X}$	SE	#	$\bar{X}$	SE	#	$\bar{X}$	SE	#	$\bar{X}$	SE	#	$\bar{X}$	SE	#	$\bar{X}$	SE	#
Progesterone	936.26	263.30	9	135.21	28.18	22	366.96	133.69	11	804.19	140.21	4	264.35	35.42	23	243.31	138.15	4	226.96	59.82	14
Pregnenolone	223.88	43.95	9	133.40	10.99	22	764.17	166.98	11	284.61	84.79	4	57.11	5.61	23	1380.92	426.83	4	83.93	16.32	14
17 α -hydroxypregnenolone	36.06	5.09	9	24.15	2.31	21	24.06	4.26	10	30.75	4.45	4	4.53	1.20	10	33.25	12.39	3	23.90	3.58	12
5 β -tetrahydrocorticosterone	1.20	0.91	3	0.31	0.28	5	0.47	0.47	9	4.21	4.21	4	1.37	0.64	8	0.00	0.00	3	0.04	0.04	11
Testosterone	11.79	3.19	9	3.34	0.87	15	1.09	0.43	6	33.21	8.80	4	15.83	2.35	22	0.00	0.00	0	11.79	2.02	13
Etiocanolone	15.68	3.67	9	12.44	1.22	22	4.57	0.56	11	14.55	3.33	4	6.86	0.68	23	9.29	0.97	4	6.33	0.98	14
Estradiol	3.88	1.40	5	1.98	0.58	8	1.86	0.98	3	12.72	2.19	4	3.86	0.80	13	5.82	2.44	3	4.49	1.31	14
Estrone	4.66	1.17	7	2.08	0.40	13	7.16	1.34	10	6.58	1.11	4	2.67	0.67	12	11.62	2.76	4	3.07	0.82	8
Androstenedione	0.16	0.16	9	0.05	0.04	13	0.07	0.06	10	0.59	0.59	4	0.23	0.11	22	0.00	0.00	4	0.03	0.02	13
5 β -tetrahydrocortisol	0.31	0.31	2	0.08	0.08	2	0.10	0.10	1	1.15	1.15	1	0.42	0.18	5	0.00	0.00	0	0.06	0.06	1
Cortisone	2.07	0.36	2	0.19	0.11	1	0.41	0.23	2	7.44	3.44	1	2.03	0.31	7	1.10	0.64	0	0.62	0.22	2
5 β -dihydrocortisone	5.06	2.54	2	3.04	1.36	1	26.94	5.15	1	22.42	5.24	1	4.62	1.49	7	23.43	8.76	0	22.30	4.95	2
Dehydroepiandrosterone	2.12	0.68	9	0.76	0.43	3	0.69	0.26	3	3.78	0.98	4	1.74	0.27	17	0.45	0.33	2	0.65	0.19	6
20 β -dihydrocorticosterone	0.06	0.06	1	0.07	0.04	3	0.05	0.05	1	0.54	0.54	1	0.08	0.04	3	2.59	2.59	1	0.00	0.00	0
5 β -tetrahydrocortisone	12.19	2.10	1	1.23	0.38	1	4.15	1.32	1	7.39	2.02	1	2.58	0.35	5	11.71	5.50	0	3.33	0.47	1
Deoxycorticosterone	0.08	0.05	5	0.01	0.01	1	0.03	0.03	2	0.09	0.09	2	0.28	0.11	21	0.00	0.00	0	0.24	0.24	4
5 β -dihydrocortisol	0.63	0.45	3	0.16	0.10	4	0.17	0.17	1	0.63	0.63	1	0.74	0.32	6	0.00	0.00	0	0.14	0.08	3
11-dehydro-tetrahydrocorticosterone	0.51	0.35	3	0.05	0.05	1	0.19	0.19	1	0.32	0.32	1	0.42	0.18	5	0.00	0.00	0	0.14	0.14	1
17 α -hydroxypregesterone	1.02	0.35	8	0.32	0.21	4	0.29	0.14	5	1.89	0.49	4	0.84	0.14	19	0.18	0.18	1	0.29	0.09	7
Dihydroprogesterone	0.39	0.30	9	0.01	0.01	9	0.10	0.09	8	0.15	0.11	4	0.15	0.06	12	0.00	0.00	2	1.25	1.21	8
5 β -dihydrocorticosterone	0.08	0.08	1	0.08	0.05	3	0.05	0.05	1	0.65	0.65	1	0.10	0.05	3	0.13	0.13	1	0.07	0.07	1
Cortisol	0.47	0.35	4	0.27	0.27	10	0.23	0.22	3	3.30	3.30	1	0.73	0.27	10	0.00	0.00	2	0.19	0.18	4
Corticosterone	0.30	0.16	1	0.23	0.09	2	0.12	0.10	2	0.53	0.53	1	0.35	0.13	6	0.10	0.06	0	0.39	0.21	2
11-ketotestosterone	0.31	0.26	2	0.03	0.02	2	0.10	0.10	1	0.20	0.20	1	0.30	0.15	5	0.00	0.00	0	0.07	0.06	2
20 β -dihydrocortisone	0.36	0.25	2	0.23	0.23	1	0.13	0.13	1	3.03	3.03	1	0.61	0.24	7	0.00	0.00	0	0.07	0.05	1
11-deoxycortisol	0.19	0.14	2	0.05	0.04	2	0.06	0.06	1	0.50	0.50	1	0.22	0.09	7	0.00	0.00	0	0.05	0.03	3
β -cortol	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0

Table 3. Principal component loadings after varimax rotation for concentrations of 27 steroid hormones quantified from the egg yolk ($n = 87$) from 7 bird species. Values in bold represent loadings >0.5 and were used to interpret each principal component

Variance explained	PC1	PC2	PC3	PC4	PC5	PC6
	9.015	3.755	3.060	2.012	1.728	1.683
Hormone						
Progesterone	0.130	0.549	0.372	-0.001	-0.145	0.336
Pregnenolone	0.051	-0.156	0.859	-0.035	-0.001	-0.088
17 α -hydroxyprogesterone	0.478	0.706	0.059	0.077	-0.118	0.318
17 α -hydroxypregnenolone	-0.048	0.178	0.673	0.029	0.196	0.389
Dihydroprogesterone	0.144	-0.007	-0.007	0.968	-0.016	0.007
Estrone	0.345	0.191	0.799	-0.099	-0.013	-0.069
Estradiol	0.185	0.522	0.255	-0.128	0.228	-0.257
Dehydroepiandrosterone	0.188	0.812	0.066	0.004	0.038	0.067
Androstenedione	0.065	0.492	0.656	0.031	-0.203	0.278
Testosterone	0.258	0.842	-0.115	-0.005	0.196	-0.027
11-ketotestosterone	0.896	0.104	0.047	0.210	-0.226	0.078
Etiocolanolone	0.088	0.243	0.086	-0.005	0.110	0.865
11-deoxycortisol	0.945	0.216	0.030	0.090	0.186	0.040
Cortisone	0.776	0.240	0.023	0.153	0.053	-0.015
5 β -dihydrocortisone	0.761	0.241	0.008	0.047	0.544	-0.018
20 β -dihydrocortisone	0.221	0.002	-0.059	0.947	0.024	-0.036
Cortisol	0.800	0.136	-0.001	0.130	-0.004	0.011
5 β -dihydrocortisol	0.933	0.113	-0.006	0.136	-0.146	0.049
5 β -tetrahydrocortisone	0.920	0.222	0.034	0.016	0.248	0.035
5 β -tetrahydrocortisol	0.920	0.212	0.046	0.003	0.284	0.022
β -cortol	0.000	0.000	0.000	0.000	0.000	0.000
Corticosterone	0.930	0.216	0.033	-0.001	0.216	0.041
5 β -tetrahydrocorticosterone	-0.071	0.074	0.590	0.151	0.242	-0.516
11-dehydrotetracorticosterone	0.916	0.095	0.061	0.024	-0.239	0.066
5 β -dihydrocorticosterone	0.712	0.169	0.138	-0.025	0.600	0.011
Deoxycorticosterone	0.471	0.696	0.059	0.069	-0.125	0.322
20 β -dihydrocorticosterone	0.173	-0.219	0.425	-0.041	0.415	0.136

Table 4. Estimated coefficients and statistical results from general linear mixed models examining relationships between principal components derived from concentrations of 27 yolk steroid hormones and attributes of seven bird species. Values in bold represent relationships that were significant at the $P<0.05$ level.

Variable	PC1		PC2		PC3	
	β (SE)	F, P	β (SE)	F, P	β (SE)	F, P
Daily survival rate	-12.47 (8.85)	1.98, 0.164	-18.04 (7.35)	6.02, 0.017	47.35 (6.32)	56.21, <0.001
Incubation length	-0.12 (0.13)	0.85, 0.361	0.06 (0.12)	0.27, 0.605	0.61 (0.11)	33.05, <0.001
Nestling length	-0.10 (0.07)	2.42, 0.124	-0.07 (0.06)	1.31, 0.256	0.25 (0.05)	24.34, <0.001
Relative egg size ^a	8.23 (4.87)	2.85, 0.097	12.65 (4.74)	7.13, 0.009	-13.21 (3.81)	12.03, 0.002
% yolk	0.06 (0.03)	3.05, 0.086	0.06 (0.03)	3.65, 0.059	-0.07 (0.02)	7.73, 0.009

^a average egg mass as a proportion of the average mass of an adult female.