Egg yolk androgen levels increase with breeding density in the European Starling, *Sturnus vulgaris*

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Egg yolk androgen levels increase with breeding density in the European Starling, *Sturnus vulgaris*

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Summary

1. High breeding density can cause elevated plasma androgen levels in adult birds. Since maternal androgens are deposited into egg yolk, high breeding density may result in elevated yolk androgen levels as well.
2. The relationship between breeding density and yolk androgen levels was examined in the European Starling, *Sturnus vulgaris*. The concentration and total content of yolk androstenedione and yolk testosterone were measured in eggs from 24 clutches distributed across nine different colonies of nestboxes.
3. Yolk androstenedione and testosterone levels were significantly higher in colonies where a greater proportion of nestboxes had active nests.
4. Yolk testosterone levels were significantly higher, and yolk androstenedione levels were marginally higher, in colonies with a greater absolute number of active nests.
5. Yolk androgen levels were not related to the number of active nests in adjacent nestboxes.
6. We conclude that female starlings nesting in colonies with higher breeding densities transfer more androgen to their eggs.
7. This relationship may be mediated by increased interfemale aggression, particularly towards floater females searching for mates or nests to brood parasitize, in high-density colonies. Such a relationship between maternal environment and maternal yolk androgens may represent adaptive maternal modification of offspring phenotype or a non-adaptive physiological constraint which females cannot avoid.

Key-words: Competition, female aggression, maternal hormones, yolk testosterone

Introduction

The phenotype of an individual depends not only on its own genes and environment, but also on the environment experienced by its mother (e.g. prenatally; Mousseau & Fox 1998). Such maternal effects may result from non-adaptive maternal responses to heterogeneous environmental stimuli. On the other hand, mothers might use information about the current environment to make predictions about the environment offspring will experience, and manipulate offspring phenotype to better fit the predicted conditions. For example, many species of insects produce winged progeny when experiencing high population density but sedentary progeny when experiencing low population density (Fox & Mousseau 1998). Winged progeny may suffer delayed reproduction and reduced potential fecundity, but these costs are compensated for by the ability to disperse to new host plants with less competition for food (Groeters & Dingle 1989). Both offspring and maternal fitness will benefit when parents prepare their offspring for the high levels of competition expected in high-density environments. We have little information regarding such maternal effects in vertebrates, although offspring phenotype is influenced by maternal density in some vertebrates (Boonstra & Boag 1987). A relationship between breeding density and transfer of maternal hormones to offspring in birds was first suggested by Schwabl (1997). We show here that some measures of breeding density experienced by female birds predict the levels of androgen hormones in their eggs. Hence, maternal transfer of hormones to offspring may provide a mechanism for density-dependent offspring phenotype manipulation in vertebrates.

High population density causes increased aggression among individuals in many vertebrate species. These effects are readily observable during the breeding season...
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in species that attempt to maintain discrete breeding territories. For example, lizards, mammals and birds living under high density or who receive repeated territorial intrusions show more intense territorial behaviour and increased likelihood of attacking intruders (Yasukawa *et al.* 1985; Wingfield & Lewis 1993; Klukowski & Nelson 1998). Aggression, in turn, exhibits a cyclical relationship with levels of androgen hormones (i.e. testosterone and related steroids) in many vertebrate taxa, where aggression leads to increased androgen levels and increased androgen levels lead to increased levels of aggression (Wingfield *et al.* 1990; Siegel & Demetrikopoulos 1993; Wingfield & Hahn 1994). Thus, it is not surprising that increased density, increased aggression and increased androgen levels are all interrelated in a variety of vertebrate species (Pankhurst & Barnett 1993; Cristol & Johnsen 1994; Chapman *et al.* 1998; Silverin 1998).

High androgen levels may be adaptive under high-density conditions, helping individuals defend or enlarge their territories and preparing them for intense competition with conspecifics (Moss *et al.* 1994).

The role of androgens in promoting aggression in female vertebrates has received less attention. Although androgen levels are usually lower in females than in males, females do have circulating androgens that vary in concentration, depending on environmental conditions (Staub & De Beer 1997). In several mammalian species, female androgen levels are responsive to received aggression and activate expression of aggression (Siegel & Demetrikopoulos 1993; Staub & De Beer 1997; Kapusta 1998). Androgens activate female aggression in lizards as well (Woodley & Moore 1999; Rhen & Crews 2000). Some female birds respond to territorial intrusions with increased androgen levels (Wingfield 1994), which activate female aggression in some, but not all, avian species (Searcy 1988; Wingfield 1994; Elekonich & Wingfield 2000). Thus, androgens mediate aggressive behaviour in females of at least some vertebrate species.

Concentrations of steroids in the maternal circulation affect steroid concentrations in eggs (Adkins-Regan *et al.* 1995; Janzen *et al.* 2002; see also: Schwabl 1996a; McCormick 1998; Lovern & Wade 2001). Variation in the maternal social environment may therefore affect egg androgen levels (see Discussion) and may have important consequences for offspring. If females breeding at high density produce more androgenic steroids than females breeding at low density, then their offspring are likely to be exposed to greater levels of yolk androgens. Exposure to greater levels of yolk androgens could encourage dispersal by offspring, and thus yolk androgens could serve as a proximate mechanism generating density-dependent dispersal. Yolk androgens are known to enhance early chick growth in several species of birds (Schwabl 1996b; Eising *et al.* 2001; Pilz 2003; but see Sockman & Schwabl 2000). Enhanced early growth may result in or be indicative of higher-quality chicks that are better able to compete with conspecifics later in life. Consequently, yolk androgens may also enhance offspring competitiveness in a density-dependent manner. Indeed, high levels of yolk testosterone are associated with success in food competition in juvenile canaries (Schwabl 1993). Thus, yolk androgens could mediate effects of maternal environment on offspring success.

We examine the relationship between breeding density and yolk androgen levels in the European Starling (*Sturnus vulgaris*). The starling is a facultatively polygynous passerine bird that breeds in natural tree cavities and in artificial nestboxes. Both males and females defend small breeding territories, primarily from same-sex conspecifics, and will attack territorial intruders (Sandell & Smith 1997). Females try to prevent their mates from attracting and mating with other females (Sandell & Smith 1996), probably because males who succeed in attracting secondary females subsequently provide less parental care at the primary female’s nest (Sandell *et al.* 1996). We predict that females breeding in high-density colonies will transfer more yolk androgen to their eggs than females who breed in low-density colonies. Previous work in this colony has shown that yolk androgens enhance growth and survival of starling chicks (Pilz 2003; K. M. Pilz, M. Quiroga, E. Adkins-Regan & H. Schwabl, unpublished observations). We find that some measures of breeding density experienced by female birds predict the levels of androgen hormones in their eggs and discuss the adaptive consequences of this relationship.

**Methods**

**STUDY SPECIES AND SITE**

We examined the relationship between breeding density and yolk androgen allocation in the European Starling (*Sturnus vulgaris, Linnaeus*) during the breeding season of 1999 (April–June). The study was conducted in the Revinge area of southern Sweden. Revinge is part of a military training area that is composed of cow-grazed pastures with small clumps of beech, birch and oak trees. Both male and female European Starlings maintain small territories immediately surrounding their nestboxes, and primarily defend their territories from intrusion by same-sex individuals (Feare 1984; Sandell & Smith 1997). The cow-grazed pastures provide excellent feeding grounds for the starlings, which typically travel from a few metres to several hundred metres from their nests to forage (Feare 1984). Population density in the pasture-rich Revinge area is high relative to the surrounding agricultural areas, as is nest site competition (H. G. Smith, unpublished data).

**BREEDING COLONIES**

All colonies used in this study had already been established for several years. The colonies consist of nestboxes mounted to trees, about 1·5–2·0 m above the ground. The nestboxes in a colony are mounted along lines of trees that border open pastures. Thus, the nestboxes...
are typically organized in a roughly linear arrangement with 6–8 m between boxes. Each nestbox has two ‘neighbouring’ nestboxes, those located adjacent to it, except for nestboxes at the end of the colony which have only one ‘neighbour’. The nestbox colonies in this study had between 14 and 19 total nestboxes (Fig. 1). The number of active nests per colony ranged from 6 to 17. The number of unused nestboxes per colony ranged from 0 to 9. Thus, colonies varied greatly in terms of competition for nestboxes. Breeding density was not determined simply by geographical location, as both high- and low-density colonies occurred on both the northern and southern sides of the study area (Fig. 1).

**YOLK ANDROGEN EXTRACTION AND ASSAY**

Steroids were extracted from a sample of approximately 150 mg yolk (weighed to the nearest 0.1 mg). Methods for yolk androgen extraction and radioimmunoassay followed those of Schwabl (1993). Briefly, tritiated steroids were added to the homogenate for calculation of steroid recovery. Steroids were extracted twice with 4 ml of petroleum ether/diethyl ether (30 : 70 vol : vol). Neutral lipids were precipitated with 90% ethanol at −20 °C. Extracts were then transferred to diatomaceous earth microcolumns for further purification and separation of steroids. Radioimmunoassays were conducted for androstenedione (A4), dihydrotestosterone and testosterone (T) following the methods of Wingfield & Farner (1975). In these analyses we only examine the androgens A4 and T, which we consider to be the most ecologically relevant in this system: A4 is the most concentrated androgen in starling eggs (Pilz et al. 2003) and yolk A4 and T are known to influence growth in starlings (Pilz 2003; K. M. Pilz, M. Quiroga, E. Adkins-Regan & H. Schwabl, unpublished observations) and other birds (Schwabl 1996b; Sockman & Schwabl 2000; Eising et al. 2001). Extraction recoveries averaged 53.4 ± 6.7% (mean ± SD) for A4 and 59.9 ± 4.6% for T; intra-assay variation was determined by females not breeding within the same colony (Sandell & Diemer 1999).

Brood parasitic eggs were identified using isoelectric focusing electrophoresis of egg albumen following the methods of Andersson & Åhlund (2001), and are discussed in detail in Pilz (2003). Six of the 24 nests received brood parasitic eggs, and these parasitic eggs were excluded from statistical analyses. In one nest we could not determine with certainty which of two eggs laid on the same day were parasitic eggs; in this case both putative parasitic eggs were excluded from the analysis.

**BREEDING DENSITY**

We measured breeding density with three indices: (1) the number of active nests per colony; (2) the proportion of nestboxes per colony which contained active nests; (3) the number of neighbouring (i.e. adjacent) nestboxes with active nests. ‘Active nests’ were any nests in which a clutch of eggs was laid and incubation begun during the synchronous laying period (25 April to 4 May). Nestboxes with only one or two eggs were not considered active nests as these clutches probably represent egg dumping. No clutches of one or two eggs were incubated.

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9.2% for A4 and 10.2% for T; interassay variation was 12.1% for both A4 and T.

STATISTICAL ANALYSES

Yolk androgen levels were not normally distributed, but log transformation \[\log_{10}(\text{yolk androgen value} + 1)\] successfully normalized the data. Log-transformed yolk androgen values were used for statistical analyses. Statistical tests were conducted with the SAS System for Windows, Version 8.01, using mixed model repeated measures ANOVA/ANCOVA. We preferred mixed models to simple or multiple regression due to the unbalanced and nested structure of our data. We tested several covariance structures for the best-fitting model in all analyses and found that compound symmetry performed best. SAS mixed models typically use the containment method to calculate the denominator degrees of freedom (ddf). Since the containment method does not perform well with unbalanced data sets, we used the Satterwaite method instead, which performs well under these conditions (Littell et al. 1996). The Satterwaite method provides a numerical approximation of ddf, so the reported ddf are not necessarily whole numbers.

We used similar models to examine the relationship of yolk androgen levels with (1) the proportion of nestboxes with active nests per colony and (2) the total number of active nests per colony. In both cases we used colony as the subject and eggs nested within nests as the repeated measure. The models used to examine the relationship of yolk androgen levels with the number of active nests in neighbouring nestboxes differed slightly. These models used nest as the subject and colony as a random factor, since different nests within a colony will have different, but not necessarily independent, numbers of neighbours. Eggs within nests were the repeated measure.

Both laying order and breeding date are known to affect yolk androgen levels in our population (Pilz et al. 2003); yolk androgen concentrations increase with laying order within clutches and decrease with breeding date (the date on which the first egg of the clutch was laid) across clutches. Therefore, we included laying order and breeding date, as well as the density measurement of interest, as predictor variables in our initial statistical models. If the effect of either laying order or breeding date did not approach significance (\(P > 0.2\)), then that factor was excluded from the model. In no case was there a near-significant interaction (\(P < 0.2\)) between any of the predictor terms, so interactions were never included in the models. In the Results section, we report the \(F\)-values, degrees of freedom and probability values for all variables that remained in the final models.

Results

Yolk androgen levels were significantly related to the proportion of nestboxes occupied within a colony (Fig. 2). Total yolk A4 content of eggs was significantly higher in colonies where higher proportions of nestboxes were occupied (\(F_{1,79} = 9.58, P = 0.015\); laying order: \(F_{5,103} = 3.84, P = 0.0031\); breeding date: \(F_{1,105} = 2.79, P = 0.098\)). Similar results were obtained when we analysed concentrations of yolk A4 (\(F_{1,74} = 10.22, P = 0.014\); laying order: \(F_{5,111} = 3.72, P = 0.0038\)). Higher total yolk content of T was also found in colonies with greater proportions of occupied nestboxes (\(F_{1,831} = 9.63, P = 0.014\); breeding date: \(F_{1,1093} = 6.65, P = 0.012\), as were higher concentrations of T (\(F_{1,16} = 10.49, P = 0.012\); breeding date: \(F_{1,106} = 4.64, P = 0.033\)).

Yolk androgen levels were moderately related to the total number of nestboxes occupied within a colony
Greater numbers of occupied nestboxes were marginally, but not significantly, associated with higher total yolk A4 contents ($F_{1,\, 82} = 4.09, P = 0.079$; laying order: $F_{5,\, 109} = 3.81, P = 0.0033$; breeding date: $F_{1,\, 109} = 3.08, P = 0.0082$) and higher yolk A4 concentrations ($F_{1,\, 49} = 4.89, P = 0.060$; laying order: $F_{5,\, 111} = 3.73, P = 0.0037$). Greater numbers of occupied nestboxes were significantly associated with higher total yolk T contents ($F_{1,\, 48} = 7.45, P = 0.028$; breeding date: $F_{1,\, 103} = 6.26, P = 0.014$) and yolk T concentrations ($F_{1,\, 44} = 8.76, P = 0.020$; breeding date: $F_{1,\, 114} = 4.23, P = 0.042$).

The yolk androgen levels of a female’s eggs were not related to the number of females breeding at neighbouring nestboxes (Fig. 4). Females with more neighbours tended to have eggs with more total yolk A4 content than females with fewer neighbours (Fig. 4a), but the difference was not significant ($F_{5,\, 88.8} = 12.02, P < 0.0001$). The number of active nests in neighbouring nestboxes was not significantly related to yolk A4 concentration ($F_{1,\, 22.1} = 2.02, P = 0.17$; laying order: $F_{5,\, 95.6} = 10.02, P < 0.0001$), total yolk T content ($F_{1,\, 22.1} = 0.44, P = 0.51$; laying order: $F_{5,\, 88.3} = 5.39, P = 0.0002$) or yolk T concentration ($F_{1,\, 22.1} = 0.48, P = 0.49$; laying order: $F_{5,\, 95.3} = 3.93, P = 0.0028$). The number of neighbouring breeding females who began laying before the focal female was also not significantly related to the concentration or total content of yolk A4 or yolk T ($P > 0.2$ in all comparisons).

**Discussion**

Our results indicate that female starlings that nest in colonies with higher breeding densities deposit more total androgen in their eggs, resulting in greater concentrations of yolk androgens. We found that a high proportion of occupied nestboxes within a colony was associated with high yolk levels of both A4 and T. The total number of females nesting in a colony was positively associated with yolk T levels, and marginally associated with yolk A4 levels. These results agree with previous findings that female environment, including breeding density, may influence yolk androgen allocation. Schwabl (1997) found that house sparrow eggs from two farm colonies, with an average internestbox distance of 5 m, had higher total testosterone contents than eggs from a residential colony, with an average internestbox distance of 15 m. Reed & Vleck (2001)
reported that female Coots nesting on ponds with three breeding pairs laid eggs with higher yolk androgens concentrations than Coots nesting on ponds with only one or two nesting pairs. Groothuis & Schwabl (2002), on the other hand, found that Black-Headed Gulls nesting in the periphery of colonies laid eggs with higher androgen levels than gulls nesting in the centre of colonies. The relationship between social competition and yolk androgen levels is unclear in this case because peripheral areas in these colonies are characterized by higher visibility and higher aggression but lower breeding density than central areas (Groothuis & Schwabl 2002). Social factors are known to play some causal role in yolk androgen allocation, as Zebra Finch females mated to apparently high-quality males deposit more androgen in their eggs than Zebra Finch females mated to apparently low-quality males (Gil et al. 1999). Maternal photoperiod also affects yolk T levels (Schwabl 1996a). Thus, there is ample evidence that maternal environment modulates yolk androgen allocation.

We considered a nest ‘active’ and included it in our density calculations if its clutch was laid any time during the synchronous breeding period. This may seem illogical to those who are not familiar with the breeding habits of our starling population, as this assumes that a female’s yolk androgen deposition could be affected by females who began laying after her. We include all active nests in our measurements for two reasons. The first is that the synchronous breeding season is so short that the laying periods of most females do overlap somewhat: the earliest clutch completion date was 30 April (seven clutches), and the latest clutch initiation date was 1 May (one clutch). With the exception of the last clutch, all clutches did overlap in laying dates. Second, females spend from several days to over a week building nests before they begin laying (K. M. Pilz & H. G. Smith, personal observation), so it would be inappropriate to consider nests inactive simply because laying had not yet begun. Furthermore, the bulk of the egg yolk, and presumably the yolk androgens, are taken up by the follicle for several days before ovulation (which occurs 1 day before laying; Burley & Vadehra 1989). Thus, any breeding female in a colony could potentially have influenced the yolk androgen allocation of any other female in that colony.

We do not know the mechanism responsible for the association between breeding density and yolk androgen levels. Since our study was correlative it does not test whether breeding density directly affects yolk androgen deposition. Instead, breeding density may be related to a third factor that has direct consequences for yolk androgen deposition. For example, the relationship between breeding density and yolk androgen deposition might have been explained as a consequence of high-quality females nesting at higher density, since we have shown previously that high-quality starling females deposit more yolk androgens in their eggs than low-quality females (Pilz et al. 2003). Nest sites are limited in particularly attractive breeding areas (H. G. Smith, unpublished data), thus only high-quality females may be able to secure nest sites in these areas. We included breeding initiation date in our statistical models to statistically control for female quality, so that this factor could not confound our analyses. Breeding initiation date is the best indicator of female reproductive success in our study area, is highly correlated with clutch size and female age (also indicators of female quality), and influences both yolk A4 and yolk T content (Pilz et al. 2003). Including female age or clutch size as covariates in our models did not change the results (data not shown). Female body condition is not related to yolk androgen levels in our population (Pilz et al. 2003). Thus, female quality is unlikely to explain the relationship between breeding density and yolk androgens found in this study.

Female–female aggression may mediate the relationship between breeding density and yolk androgens (see Introduction). Individuals breeding at high density generally show increased aggression (Wingfield & Lewis 1993), and breeding female starlings are aggressive towards females intruding on their territories (Eens & Pinxten 1996; Sandell & Smith 1997). Increased aggression can lead to elevated circulating levels of androgens (Wingfield 1994), indicating increased androgen synthesis. Ovarian follicles are a primary site of androgen synthesis in female birds (Balthazart et al. 1986; Schlinger et al. 1999). Since the yolk develops in the ovarian follicle, increased androgen synthesis will probably lead to elevated yolk androgen levels, even if circulating androgen levels themselves do not increase (see Hackl et al. 2003). Indeed, Whittingham & Schwabl (2002) have recently reported a positive correlation between the number of aggressive interactions that a female Tree Swallow engages in and the level of yolk testosterone in her eggs.

If aggression does mediate the relationship between breeding density and yolk androgens, with whom are females engaging in aggression? Our three measures of breeding density differed in their degree of relatedness to yolk androgen levels. From these differences we can attempt to infer how aggression might mediate the observed yolk androgen effects. Interfemale aggression frequently occurs near nestboxes (Eens & Pinxten 1996). However, aggressive interactions with neighbouring females do not appear responsible for the relationship, since the number of active nests in adjacent nestboxes was not related to yolk androgen levels. Aggressive interactions with females breeding in the same colony may explain the relationship. Females breeding in high-density colonies probably interact with other breeding females more frequently, and thus may engage in more frequent interfemale aggression. On the other hand, effects of density on yolk androgens may be mediated through aggression with unmated female floaters. Starling females are aggressive towards female floaters near their nestboxes or attempting to pair with their mates (Eens & Pinxten 1996; Sandell
Floating females may settle in colonies as secondary females, and secondary females cause a decrease in paternal care devoted to the clutches of primary females (Sandell et al. 1996). Furthermore, floating females may brood parasite breeding females (Sandell & Diemer 1999). Floating females thus threaten the fitness of mated females, so it is not surprising that mated females are aggressive towards them. As more floating females exert pressure to establish breeding sites within a colony, success of established females in preventing their settlement will probably decline, and the proportion of occupied nestboxes should increase. Thus, the proportion of occupied nestboxes in a colony may represent the pressure that unmated females are exerting to settle in a colony, i.e., competition for nest sites. The fact that the proportion of occupied nestboxes significantly affected A4 levels, whereas the absolute number of occupied nestboxes only marginally affected A4, may indicate that the relationship between breeding density and yolk androgen levels are mediated by floater-directed aggression, not aggression oriented toward established females breeding in the same colony. However, at this point the relative roles of aggression vs neighbours, vs colony-mates or vs floaters in mediating yolk androgen deposition are speculative; more research on the effects of aggression and breeding density on yolk androgen levels is needed.

If females breeding in high-density colonies deposit high levels of androgen in their eggs as a result of other physiological or ecological mechanisms, such as aggression-induced androgen synthesis, then depositing high levels of yolk androgens when breeding in high-density colonies may not be adaptive. In fact, the trait could be maladaptive, since yolk androgens can be detrimental to offspring fitness in some species (Sockman & Schwabl 2000). One could imagine that the cost to females of avoiding aggression might be greater than the cost of elevated yolk androgen levels in her eggs, so that the female cannot avoid the associated fitness loss. In this case, high yolk androgen levels in eggs of females breeding at high density would represent a physiological constraint. Furthermore, it is also possible that breeding density affects yolk androgen levels without affecting circulating maternal androgen levels. This could be accomplished if breeding density modifies transport of steroids from the ovary to the yolk, though such a mechanism is unlikely since steroids passively diffuse through biological membranes.

We proposed in the Introduction that a relationship between breeding density and yolk androgen levels may represent adaptive maternal modification of offspring phenotype, wherein avian mothers prepare offspring for high-density, high-competition environments. Such a mechanism could be general across birds, since there is evidence from several species for a positive relationship between breeding density and yolk androgen levels (Schwabl 1997; Reed & Vleck 2001; this study). For example, elevated yolk androgen levels could encourage offspring dispersal, producing an effective transgenerational mechanism for density-dependent dispersal. In bird species where breeding density predicts the level of offspring competition at fledging or at adulthood, the relationship between breeding density and yolk androgen levels may be adaptive if yolk androgens enhance offspring competitiveness. Breeding density can be expected to predict offspring competition in species that defend feeding territories, as occurs in many avian species. It is not clear how such a mechanism would function in species such as starlings, which do not forage on their territories and may not compete directly for food. One possibility is that colony breeding density is correlated to local population density, and therefore carries information about future population-level competition. Advantages bestowed upon chicks by high levels of yolk androgens include increased growth (Schwabl 1996b; Eising et al. 2001; Pilz 2003). Increased growth might be advantageous in conditions of high density, because rapid growth may allow offspring to fledge larger or sooner than other fledglings. This may enhance the fledging’s ability to compete for food and to attain sufficient body condition for migration. Furthermore, high yolk androgen levels are associated with success in food competition trials in juvenile canaries (46–131 days old; Schwabl 1993). Yolk androgens could also affect the organization of adult traits and enhance competitive success in adults (e.g. for food, territories or mates), thereby increasing offspring and maternal fitness. Thus, yolk androgens may provide a mechanism by which mothers breeding at high density prepare offspring for elevated competition. Our correlative study does not prove that variation in breeding density itself causes variation in yolk androgen levels. However, density need not directly cause elevation in yolk androgen levels for this mechanism to function; such a relationship could be adaptive as long as high density is associated with yolk androgens (e.g. because both are associated with high levels of aggression). The enticing hypothesis that avian mothers differentially allocate yolk androgens to prepare offspring for future competition warrants further testing in starlings and other species.

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