

Yolk testosterone and estradiol variation relative to clutch size, laying order and hatching asynchrony in Common Grackles

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Abstract Maternal effects are typically thought to enhance rather than reduce offspring performance, but asynchronous hatching (ASH) in birds typically produces a size hierarchy within a clutch that frequently reduces the growth and survival of nestlings from eggs that hatch later. Given that yolk steroids can significantly affect offspring phenotype and that in many species the levels of yolk steroids have been found to increase with laying order, the maternal transfer of steroids to egg yolk has been proposed as a mechanism for females to offset the deleterious effects of ASH. To test this hypothesis, we determined whether yolk steroids varied with laying order or clutch size in Common Grackles (*Quiscalus quiscula*). Because ASH varies with clutch size (hatching span averages 48 h in five-egg clutches, 24 h in four-egg clutches) and regularly results in the starvation of later hatched nestlings, we predicted: (1) testosterone and 17 β -estradiol levels should increase with laying order in both clutch sizes to mitigate the negative effects of ASH on last-hatched nestlings, and (2) the increase should be greater in five-egg clutches due to more pronounced hatching asynchrony. Using a competitive-binding steroid radioimmunoassay, we found no systematic variation in either testosterone or estradiol levels relative to laying order or clutch size. In the absence

of evidence that yolk steroids interact adaptively with ASH, research must look elsewhere for potential benefits that might compensate for the costs these steroids impose on nestlings.

Keywords Asynchronous hatching · Estradiol · Maternal effects · Testosterone

Introduction

Asynchronous hatching (ASH) in birds results from females beginning incubation before clutch completion and typically produces a size hierarchy among nestlings within a brood (Clark and Wilson 1981; Magrath 1990; Stoleson and Beissinger 1995). Consequently, older nestlings are afforded a considerable size and strength advantage that frequently results in the starvation of younger nestlings. There are additional mechanisms by which females can either further enhance or ameliorate the effects of the size hierarchy resulting from ASH; these include varying egg size relative to laying order (especially the size of the last egg), which enables a female to affect the size and strength disadvantage of last-hatched nestlings (Slagsvold et al. 1984). A female may also be able to influence competition among nestlings by varying the amount of androgen she deposits in each egg (Schwabl 1993; Winkler 1993; Eising et al. 2001; Sockman et al. 2006). Here we explore patterns of yolk steroid deposition in eggs of Common Grackles (*Quiscalus quiscula*), a blackbird (Icteridae) species that is abundantly distributed across the eastern and central USA and Canada (see Peer and Bollinger 1997 for further information) and which exhibits both pronounced ASH and egg-size variation with laying order (Howe 1976, 1978).

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Since steroids in the avian egg yolk were first shown to be of maternal origin (Schwabl 1993), numerous studies have demonstrated that the concentration of yolk androgens can affect offspring phenotype (reviewed by Gil 2003; Groothuis et al. 2005). Several phenotypic traits that are modifiable by yolk androgens can affect nestling performance. Lipar and Ketterson (2000) experimentally and Lipar (2001) descriptively demonstrated that the relative mass of the hatching muscle (*musculus complexus*) of Red-Winged Blackbird (*Agelaius phoeniceus*) and European Starling (*Sturnus vulgaris*) nestlings, respectively, was positively correlated with yolk testosterone. Because the hatching muscle is used to break open the shell at hatching and in begging for food, a larger hatching muscle may increase the competitiveness of nestlings by decreasing hatching times and enhancing begging effectiveness. In the domesticated canary (*Serinus canaria*), nestlings that hatched from eggs that had experimentally increased concentrations of testosterone exhibited enhanced growth and begging frequency within 22 h of hatching (Schwabl 1993). Similarly, Black-Headed Gull (*Larus ridibundus*) eggs injected with testosterone produced nestlings that hatched earlier than controls, begged more often and were fed more by their parents (Eising et al. 2001; Eising and Groothuis 2003).

Consistent with the possibility of yolk androgens being used by females to modify the effects of ASH, deposition patterns often vary with laying sequence. In some species, androgens increase with laying order (Schwabl 1993, 1996; Lipar et al. 1999; Sockman and Schwabl 2000; Eising et al. 2001; Mazuc et al. 2003), potentially providing late-hatched nestlings some competitive advantage. In other species, however, androgens decrease with laying order (Schwabl et al. 1997; Gil et al. 1999; Reed and Vleck 2001), potentially compounding the competitive disadvantage of late-hatched nestlings. Few studies have been designed to explicitly determine how patterns of yolk hormone allocation are related to the degree of hatching asynchrony. Consequently, it remains uncertain whether the patterns of hormone allocation are adaptive (i.e. have been favored by selection because of their effects on nestling performance) or whether they are simply a passive consequence of breeding female physiology (Sockman et al. 2006).

Female birds may also be able to modify offspring performance by altering the levels of yolk estradiol (Adkins-Regan et al. 1995; Williams 1999; Williams et al. 2004). Yolk estradiol has been considerably less studied than androgens, but similar to androgens, estradiol can increase or decrease over the laying sequence. Williams et al. (2005) found that estradiol levels in Zebra Finch (*Taeniopygia guttata*) clutches decreased with laying order in control females and increased with laying order when

females were injected with estradiol. Similarly, Elf and Fivizzani (2002) found that estradiol concentrations tended to decline over the laying sequence in Leghorn Chickens (*Gallus domesticus*). Limited evidence also suggests that estradiol can affect offspring growth. Zebra Finch nestlings of estradiol-treated females hatched earlier (approximately 12 h) and weighed more at the midpoint of the nestling stage than nestlings of the control group (von Engelhardt et al. 2004).

Indirect evidence that female grackles may vary yolk hormones with laying order is already available. At different study sites in Midwest USA, Howe (1976) and Maddox and Weatherhead (2008) forced females with five-egg clutches to start incubation after clutch completion, thereby largely eliminating hatching asynchrony. Eggs were removed from nests on the day they were laid and replaced with artificial eggs. Real eggs were maintained at 10–15°C to suspend embryo development and returned to their original nest once egg laying was completed. Nestlings from control nests predominantly hatched in the order in which they were laid, whereas the hatching order was completely reversed in synchronized nests (i.e., last-laid eggs hatched first). A plausible explanation for the pattern in synchronized nests is that yolk hormone concentrations increased with the laying order in five-egg clutches, causing accelerated development in the eggs laid later.

In the study reported here, we examined variation in yolk steroids both within and among grackle clutches. In this species, ASH and egg-size variation relative to laying order are both substantial and vary with clutch size (Howe 1976, 1978). In five-egg clutches, mean egg size increases by 9% from the first- to last-laid eggs, and last-laid eggs hatch on average 24–48 h after first-laid eggs. In contrast, in four-egg clutches, egg size does not increase with laying order, and hatching asynchrony averages only 24 h (Howe 1978). The size hierarchy produced by ASH means that the competitive disadvantage of last-hatched nestlings is severe. Indeed, on average, approximately 20% of all nestlings starve in four- and five-egg clutches (Howe 1978), mainly because last-hatched nestlings are so much smaller than their siblings at the completion of hatching (Maddox and Weatherhead 2008). We tested two predictions. First, if female grackles vary the amount of steroids they deposit in their eggs to affect the competitive ability of their nestlings (i.e., ameliorate the disadvantages of ASH), then steroid levels should increase with laying order in both four- and five-egg clutches. Second, due to the greater hatching asynchrony of five-egg clutches, steroid levels should increase with laying order at a greater rate in five- than in four-egg clutches (i.e. we should find a significant interaction between laying order and clutch size).

Methods

We collected eggs from Common Grackle nests between 13 April and 28 April 2006 from a colony nesting on a Christmas tree farm near Mansfield, Illinois, USA (40°13'N, 88°28'W). Nests were found prior to egg laying and checked daily to determine clutch initiation. Eggs were removed on the day they were laid, uniquely marked and replaced with wooden eggs of similar size and appearance to prevent female abandonment before clutch completion. At the time of removal, each egg was weighed to the nearest 0.1 g, measured (length and width) to the nearest 0.1 mm and frozen at -20°C until hormone analysis.

Sample preparation and radioimmunoassay

Samples were prepared by carefully breaking open and separating the entire yolk from the shell and albumen while the yolk was still frozen. Frozen whole yolks were weighed to the nearest 0.001 g and homogenized. Approximately 10 mg homogenized yolk (range 9–12) was removed from each sample and suspended in 500 μL dH_2O .

To measure the concentration of testosterone and 17β -estradiol, we used a competitive-binding steroid radioimmunoassay (RIA) following the protocol of Wingfield and Farner (1975) as modified for yolk assays by Schwabl (1993) and Bowden et al. (2000). We added 2000 cpm of tritiated testosterone and 17β -estradiol (Perkin-Elmer, Foster City, CA) as a tracer before vortexing and allowing all samples to equilibrate overnight at 4°C . Hormones were extracted from samples using petroleum and diethyl ether; samples were then dried, reconstituted in 90% ethanol and after sitting overnight, dried again and re-suspended in 10% ethyl acetate in isooctane for column chromatography. Samples were applied to columns consisting of a celite-glycol mixture upper phase and a celite-water lower phase, and hormones were eluted (20% ethyl acetate: isooctane for testosterone; 40% ethyl acetate:isooctane for 17β -estradiol). The fractionated samples were dried and re-suspended in assay buffer (PBSg). Duplicate samples were assayed for testosterone and 17β -estradiol using specific antibodies (testosterone antibody from Research Diagnostics, San Antonio, TX; 17β -estradiol antibody from Biogenesis, Poole, Dorset, UK).

Four separate assays were required to analyze the samples. All eggs from the same clutch were included in the same assay but randomized with respect to position in the assay, and clutches of a given size were spread across assays. Recovery values, averaged across assays, were 71% for testosterone and 63% for 17β -estradiol. Intra-assay variation was 7.2, 6.7, 3.5, and 6.4% for testosterone and 1.7, 6.1, 3.1, and 5.7% for 17β -estradiol, while inter-assay variation was 13.3 and 12.4%, respectively.

Data analysis

To determine the effects of hatching asynchrony and egg size, we constructed linear mixed-effects (LME) models (LME, package NLME; Venables and Ripley 2002) in R 2.6.1 (R Development Core Team 2008) using testosterone and estradiol as response variables. Initial models contained egg mass, clutch size, laying order and the interaction between clutch size and laying order as fixed effects of interest. Because yolk mass and egg mass were positively correlated ($F_{1,178} = 67.13$, $R^2 = 0.27$, $P < 0.001$), only egg mass was included in the models. The results of the models were the same if egg mass was replaced with yolk mass. Nest was included as a random effect in all models to account for non-independence of eggs within the same clutch (Krackow and Tkadlec 2001). Final models were determined by removing all terms that did not contribute significantly to the model fit (Crawley 1993).

Results

We collected 180 eggs from 39 clutches: 15 four-egg and 24 five-egg clutches. Egg mass increased with laying order in five-egg clutches ($F_{1,118} = 6.244$, $R^2 = 0.05$, $P = 0.01$), but not in four-egg clutches ($F_{1,58} = 0.17$, $R^2 < 0.01$, $P = 0.68$; Fig. 1). Neither estradiol nor testosterone concentrations (ng g^{-1} yolk) varied with any of the fixed effects of interest. Thus, the interaction terms were not significant, and the null models were retained. Results from the LME models are supported by univariate tests. Testosterone did not increase with laying order in four-egg ($F_{1,58} = 0.11$, $R^2 < 0.01$, $P = 0.74$) or five-egg clutches

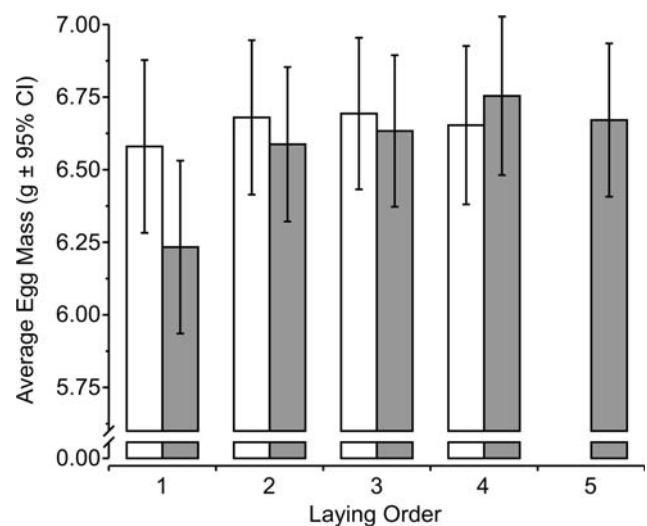


Fig. 1 Mean egg mass [$\text{g} \pm 95\%$ confidence intervals (CI)] relative to laying order in four-egg (open bars) and five-egg (gray bars) clutches

($F_{1,118} = 1.09$, $R^2 < 0.01$, $P = 0.30$; Fig. 2). The pattern in five-egg clutches (Fig. 2) suggests that testosterone might have increased in a step-wise rather than a linear fashion, but mean testosterone levels in the first two eggs were not significantly different from those in the last three eggs ($F_{1,118} = 0.37$, $P = 0.54$). Estradiol did not increase with laying order in either clutch size (four-egg clutch: $F_{1,58} < 0.01$, $R^2 < 0.01$, $P = 0.96$; five-egg clutch: $F_{1,118} = 0.37$, $R^2 < 0.01$, $P = 0.55$; Fig. 3). Neither estradiol nor testosterone concentrations were different among clutch sizes (estradiol: $F_{1,37} = 0.96$, $P = 0.33$; testosterone: $F_{1,37} < 0.01$, $P = 0.96$; Fig. 4).

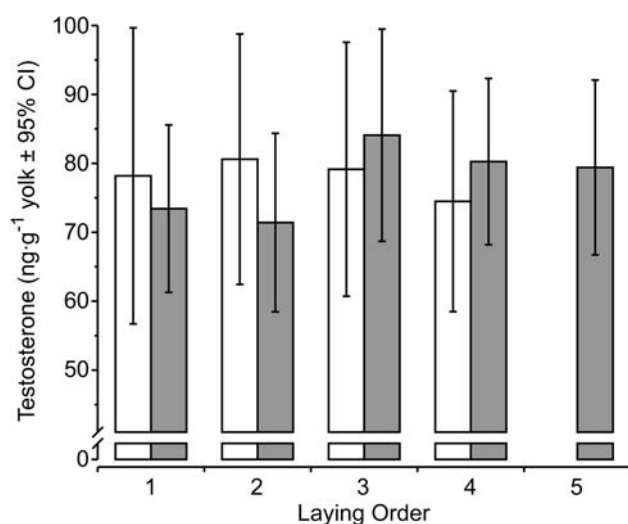


Fig. 2 Mean testosterone concentration (ng g^{-1} yolk \pm 95% CI) relative to laying order in four-egg (open bars) and five-egg (gray bars) clutches

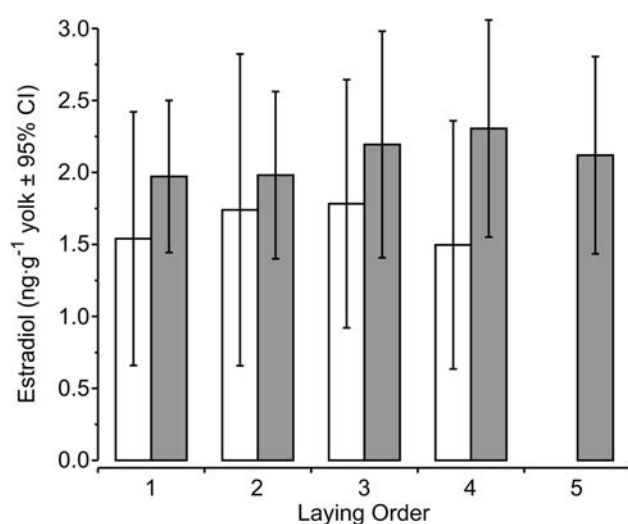


Fig. 3 Mean estradiol concentration (ng g^{-1} yolk \pm 95% CI) relative to laying order in four-egg (open bars) and five-egg (gray bars) clutches

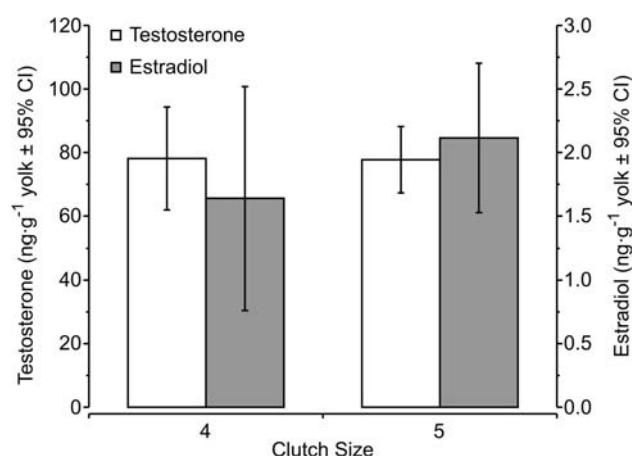


Fig. 4 Mean clutch testosterone and estradiol concentration (ng g^{-1} yolk \pm 95% CI) in four-egg (open bars) and five-egg (gray bars) clutches

Discussion

If yolk steroids do offset the deleterious effects of ASH on nestling growth and survival, we predicted that steroid concentrations should increase with laying order in both four- and five-egg clutches. We also predicted that the rate of increase would be greater in five-egg clutches, because higher levels of yolk steroids would be necessary to offset the greater hatching asynchrony in five-egg clutches. We did not find any association between laying-order and testosterone or estradiol concentrations for either clutch size, nor did we find differences in concentrations of either hormone between four- and five-egg clutches. Therefore, despite grackles appearing to be good candidates in which to find adaptive patterns of maternal allocation of yolk steroids and contrary to available research, we found neither of the patterns we predicted nor any laying order–yolk steroid pattern at all.

Even had we found higher steroid levels in eggs laid later, however, it is not clear that those steroids could have compensated for the severe disadvantage of hatching late. Schwabl (1993) first suggested that increasing levels of testosterone with laying order in canaries might mitigate the negative effects of ASH on last-hatched nestlings. However, Schwabl (1996) later refuted his initial hypothesis when further research on the same population of canaries revealed that hatching asynchrony overwhelmed the positive effects of yolk androgens on nestling growth, even though last-laid eggs had nearly fourfold more testosterone than first-laid eggs. He found that in the absence of hatching asynchrony, 10-day old nestlings that hatched from eggs containing higher concentrations of testosterone (i.e. later laid eggs) were 22% heavier than their siblings. However, with normal hatching asynchrony (i.e. 6–33 h), earlier hatched nestlings weighed 15% more than their

siblings despite hatching from eggs with less testosterone. These results led Schwabl to conclude that testosterone concentrations cannot compensate for the growth differences produced by ASH, although he saw the potential for testosterone to provide a mechanism for parental favoritism in species lacking ASH.

Subsequent studies that have found a positive relationship between laying order and yolk androgens in species that hatch their eggs asynchronously (see reviews by Gil 2003; Groothuis et al. 2005; Sockman et al. 2006) almost invariably invoke Schwabl's initial argument that yolk androgens are likely to compensate for the effect of ASH. However, only a few studies have determined the extent of any compensation using Schwabl's (1996) experimental approach—i.e. following the performance of nestlings from eggs in which yolk samples were obtained non-destructively. We are aware of only two field studies that were specifically designed to address the effects of both yolk androgens and ASH on nestling growth and survival (but see Müller et al. 2004 for a similar study that does not address nestling performance). In a study of House Wrens (*Troglodytes aedon*), Ellis et al. (2001) predicted that yolk androgens would increase with laying order in asynchronously hatching nests but not in synchronous nests because their previous research (Harper et al. 1992, 1994) indicated that late-hatched nestlings were of a similar quality in both synchronous and asynchronous nests. However, these researchers found no laying-order pattern in total yolk androgen concentrations in either synchronously or asynchronously hatching nests. In contrast, in a study controlling for egg quality and hatching asynchrony, Eising et al. (2001) found that increasing androgen (testosterone and androstenedione) concentrations of last-laid Black-Headed Gull (*Larus ridibundus*) eggs increased nestling size and weight. However, the survival of androgen-treated nestlings was unchanged, and hatching asynchrony was the most important determinant of the size hierarchy within broods regardless of treatment (i.e. androgen vs. control).

It is clear that in some species yolk steroids can enhance nestling performance, and it is conceivable that to some extent yolk steroids could mitigate the size hierarchies produced by ASH. Nevertheless, evidence that yolk steroids can offset the adverse effects of ASH is inconsistent across species. Although some experimental studies have found that the growth of nestlings from last-hatched eggs can be enhanced in species with ASH, that enhancement was not sufficient to affect survival (Schwabl 1996; Eising et al. 2001). The association between yolk hormones and ASH appears to be similar to that between egg size and ASH. Although it has been proposed that positive effects of egg size could offset the disadvantages of ASH on last-hatched nestlings (Slagsvold et al. 1984), recent evidence from Common Grackles

shows that ASH overwhelms any positive egg-size effects (Maddox and Weatherhead 2008). Thus, egg size is likely to be important only when hatching asynchrony is either greatly reduced or absent, which coincidentally is essentially Schwabl's (1996) argument regarding yolk hormones. Consequently, before invoking compensatory effects of yolk androgens against ASH, non-destructive sampling (see Lipar and Ketterson 2000; Schwabl 1993) and/or experimental manipulations (see Eising et al. 2001) that allow an individual to be followed from egg to fledgling should be undertaken so any compensatory effects can be quantified. As well, there is a need to determine both the causes and consequences of variation in yolk steroids in species for which that variation appears to have no adaptive value (Marshall and Uller 2007).

Zusammenfassung

Unterschiede im Testosteron- und Estradiolgehalt im Dotter des Purpur-Grackel in Bezug auf Gelegegröße, Legereihenfolge und Schlupf-Asynchronität

Generell geht man davon aus, dass maternale Effekte die Qualität der Nachkommen eher verbessern als reduzieren. Eine Asynchronität im Schlupf (ASH) führt bei Vögeln aber normalerweise zu einer Größenhierarchie innerhalb eines Geleges, wodurch häufig die Wachstumsrate und die Überlebenswahrscheinlichkeit von Nestlingen aus später geschlüpften Eiern reduziert ist. Vor dem Hintergrund, dass Steroide im Dotter den Phänotyp der Nachkommen stark beeinflussen können und dass sich bei vielen Vogelarten ein Anstieg des Steroidgehalts im Dotter mit der Legereihenfolge beobachten ließ, betrachtete man die maternale Übertragung von Steroiden ins Eidotter als Instrument der Weibchen, um die nachteiligen Effekte der ASH auszugleichen. Um diese Hypothese zu testen, untersuchten wir, ob bei Purpur-Grackeln (*Quiscalus quiscula*) der Steroidgehalt im Dotter mit der Legereihenfolge oder der Gelegegröße variiert. Weil die ASH mit der Gelegegröße schwankt (die Zeitspanne für den Schlupf beträgt bei Gelegen von fünf Eiern durchschnittlich 48 h, bei vier Eiern 24 h) und in der Regel zum Verhungern später geschlüpfter Nestlinge führt, trafen wir folgende Voraussagen: (1) Der Testosteron- und 17- β -Estradiol-Gehalt sollte mit der Legereihenfolge in beiden Gelegegrößen ansteigen um die negativen Effekte der ASH auf die zuletzt geschlüpften Nestlinge zu reduzieren, und (2) der Anstieg sollte in Gelegen mit fünf Eiern größer sein, da dort die Schlupf-Asynchronität stärker ausgeprägt ist. Mit Hilfe eines Competitive Binding Steroid-Radioimmunoassays konnten wir keinerlei systematische Schwankungen im Testosteron- oder Estradiolgehalt in Bezug auf die Legereihenfolge oder

die Gelegegröße finden. Ohne den Nachweis, dass Steroide im Dotter adaptiv mit der Schlupf-Asynchronität wechselwirken, muss man nach möglichen Vorteilen, die die Kosten von solchen Steroiden für die Nestlinge aufwiegen könnten, woanders suchen.

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