

Are yolk androgens and carotenoids in barn swallow eggs related to parental quality?

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Abstract Recently, evidence is mounting that females can adaptively engineer the quality of their offspring via the deposition of yolk compounds, including carotenoids and androgens. In this study, we simultaneously consider how both carotenoids and androgens in egg yolk relate to parental quality in barn swallows (*Hirundo rustica erythrogaster*). First, we found no relationship between concentrations or amounts of yolk androgens and carotenoids. Yolk carotenoids decreased with laying order, whereas we found no relationship between yolk androgens and laying order. Second, we tested the Investment Hypothesis, which predicts that high-quality females or females paired to high quality mates, allocate differentially more of these yolk compounds to their offspring. For carotenoids, we mostly found evidence to counter predictions of the Investment hypothesis: (1) Carotenoid concentrations varied among females, (2) heavier eggs contained lower carotenoid concentrations, although

heavier yolks contained greater amounts of carotenoids, (3) eggs of earlier-laying females had lower concentrations in their eggs, and (4) yolk carotenoids were not correlated with clutch size or male plumage ornamentation. For androgens, we found weak support for the Investment Hypothesis: (1) Yolk androgens varied among females, (2) heavier eggs and yolks contained greater amounts, although not concentrations of androgens, (3) females paired to more colorful males laid eggs with greater concentrations of androgens, and (4) no effects of laying date or morphological correlates of female quality on androgen concentrations in egg yolks. Overall, these findings suggest that each yolk compound may have different functions and therefore may be regulated by different mechanisms.

Keywords Barn swallow · *Hirundo rustica* · Investment hypothesis · Maternal effects · Yolk androgens · Yolk carotenoids

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Introduction

Animal parents can influence the quality of their offspring in a variety of genetic and nongenetic ways. Pre-embryonic “maternal effects” are one of several nongenetic means of parental care and can include the deposition of antibodies, nutrients, and hormones that may affect offspring development. For example, avian egg yolk contains androgenic hormones (Schwabl 1993, 1997, reviewed in Groothuis et al. 2005) and carotenoids (e.g., Royle et al. 1999; Bortolotti et al. 2003) of maternal origin, and researchers have proposed adaptive explanations for the function of these compounds in terms of offspring survival and performance (androgen: Schwabl 1996; Lipar and Ketterson 2000; Eising et al. 2001; Pilz et al. 2004, reviewed in Groothuis et al.

2005; Eising et al. 2006, but see Sockman and Schwabl 2000; Müller et al. 2005; Navara et al. 2005; carotenoids: Surai et al. 2001; Saino et al. 2003; McGraw et al. 2005).

Many studies of birds have revealed that these yolk compounds vary in eggs among females and across the sequence of eggs laid in a clutch, indicating that some females are able to allocate more or less than others (androgens: e.g., Schwabl 1993; Lipar et al. 1999; Pilz et al. 2003; Gil et al. 2005; carotenoids: e.g., Royle et al. 1999, 2001; Saino et al. 2002, 2003; McGraw et al. 2005). Moreover, measures of maternal quality (e.g., clutch size, egg and/or yolk mass, or laying date) have been shown to be positively related to yolk androgen content (Pilz et al. 2003; Gil et al. 2005), which suggests that the deposition of these compounds is a function of a female's condition or reproductive experience. Further, females who perceive their mates as high quality allocate more androgen (Gil et al. 1999; Gil et al. 2004; Tanvez et al. 2004; but see Marshall et al. 2005; Navara et al. 2006; reviewed in Groothuis et al. 2005) but, interestingly, lower amounts of carotenoids (Saino et al. 2002) to their eggs. These patterns, coupled with some emerging evidence that androgens and carotenoids are costly for females to provide to egg yolk (e.g., Blount et al. 2002; McGraw et al. 2005; but see Royle et al. 2003), suggest that these yolk compounds represent a form of adaptive maternal investment in offspring (e.g., Schwabl 1993; Gil et al. 1999; Pilz et al. 2003).

The degree to which these two classes of molecules exhibit similar patterns in eggs or have similar fitness effects within an individual or species, however, has largely been neglected. Androgens traditionally are thought to elevate competitiveness and growth rate but may also be immunosuppressive (reviewed in Groothuis et al. 2005; Ketterson and Nolan 1999) and cause oxidative damage through their enhancement of growth. Carotenoids are antioxidants and can be beneficial immunologically (e.g., Krinsky 2001; McGraw and Ardia 2003; Saino et al. 2003). Thus, there is the potential for a complementary association between these two compounds in yolk, with females laying eggs with high amounts of both in a yolk in order for carotenoids to offset the effects of androgens on immune function or androgen-enhanced fast growth. However, to date, only a few studies that have simultaneously examined the relationships of these two yolk compounds, and the results are not consistent among them. For example, several studies on lesser blacked gulls (*Larus fuscus*) found that eggs with greater concentrations of androgens typically had lower concentrations of carotenoids (Royle et al. 2001; Verboven et al. 2005; Groothuis et al. 2006), although it is important to note that Verboven et al. (2005) also determined that changes in carotenoid concentrations are not always coupled with changes in yolk testosterone concentrations. It is interesting to note, however, that

Navara et al. (2006) reported that concentrations of yolk antioxidants (including several carotenoids and four variants of vitamin E) were positively correlated with total amounts of yolk androgens in the eggs of house finches (*Carpodacus mexicanus*). Therefore, there is evidence of both a complementary and positive association between these yolk compounds, in addition to other possible relationships including the idea that females may allocate different amounts of carotenoids and androgens to their eggs to optimize growth, health, and competitiveness for each offspring (e.g., Groothuis et al. 2006). Therefore, without a priori prediction of the nature of an association between these yolk compounds, we first set out to explore whether or not they were correlated with one another within clutches and among different females in our study area.

A second objective of our study was to gain an understanding of how aspects of parental quality, including a female's phenotype and other predictors of seasonal reproductive success that have previously been shown to affect the yolk levels of each compound singly, are related to the deposition of yolk carotenoids and androgens. Specifically, we tested predictions of the Investment Hypothesis (see Table 1), which postulates that females of high quality and females paired to high-quality males should allocate more yolk compounds, such as carotenoids and androgens, to their eggs. Although the predictions of this hypothesis are simplistic because they assume that more of a compound is better, we do not have enough information to a priori make more biologically accurate and perhaps more complex predictions for patterns of allocation (e.g., that specific yolk compounds are allocated in combinations that are optimized for lay order and/or environmental variables). Moreover, testing these simple predictions allows for comparisons among various studies that have also looked for similar relationships (e.g., relationships between yolk compounds and attributes of parents: e.g., Pilz et al. 2003; Navara et al. 2006).

It is interesting to note that there is also current debate about whether patterns of allocation by females is a function of male quality because of investment (the differential allocation hypothesis) or compensatory strategies (reviewed recently by Moreno-Rueda 2007); both of which predict high levels of investment to high-quality and low-quality males. As such, we provide two plausible patterns for relationships between female allocation and male quality in Table 1.

Materials and methods

We studied Barn Swallows *Hirundo rustica erythrogaster* at ten breeding colonies in Tompkins County, NY. With permission from the US Fish and Wildlife Service and the

Table 1 Predictions of the investment hypothesis (Gil et al. 1999; Pilz et al. 2003) and results of our study

Variables examined	Predicted patterns	Results	
		Carotenoids	Androgens
Variation among females	A prerequisite of the Investment Hypothesis is that females deposit different amounts of yolk compounds to their offspring	Yes	Yes
Concentration/amount by egg mass	Females who lay large eggs will deposit greater concentrations/total amounts of androgens and carotenoids in their eggs	Lower total concentrations in large eggs Greater total amounts in large yolks	Greater total amounts in large eggs and yolks
Concentration/amount by clutch size	Females who lay large clutches will deposit greater concentrations/total amounts of yolk compounds compared to females who lay smaller clutches	No pattern	No pattern
Concentration/amount by lay dates	Early-laying females will deposit greater concentrations and amounts of yolk compounds compared to later-breeding females	Lower concentrations in early-laid eggs	No pattern
Concentration/amount by female quality	Eggs from higher-quality females will have greater concentrations and total amounts of yolk compounds	Lower concentrations from high-quality females	No pattern
Concentration/amount by male quality	Eggs from females paired to high-quality males (via differential allocation) or low-quality males (via compensatory strategies) will deposit greater concentrations and total amounts of yolk compounds	No pattern	Greater concentrations when paired to high-quality male

We specifically use the terms “concentrations” and “total amounts” to distinguish results based on these measurements.

New York Department of Environmental Conservation, we collected eggs as they were laid until clutches were completed (range in clutch size=2–6 eggs, mean=4.91 eggs, SD=0.65) from 23 barn swallow nests. As it was collected, each egg was replaced by a plastic egg that matched barn swallow eggs in appearance and weight. The replacement of real eggs with model eggs ensured that each clutch was not abandoned during the laying period (none were) and that a complete clutch of eggs was laid. In all cases, females continued to lay a normal clutch size and proceed to initiate the incubation period despite the fact that her nest was full of plastic eggs by the end of the collection phase. Eggs were collected on the morning of laying, kept cool throughout the day, and brought to the laboratory in the same evening when total egg mass (wet) was measured. The yolk of each egg was removed and homogenized, and aliquots of yolk were diluted and frozen at -20°C . Egg collection took place during the early part of the breeding season (in May–June 2002), when natural clutch losses commonly occur because of abandonment in cold weather or predation and are rapidly replaced with a new clutch of eggs.

Both the social mother and father at 21 of these nests were captured and individually marked. At this time, we also measured plumage color (see the methods below) and tail-streamer length, two traits that are associated with seasonal reproductive success in our study population

(Safran and McGraw 2004; Safran et al. 2005) or populations of barn swallows in Europe (e.g., Saino et al. 1997).

Plumage color scoring Although color throughout the ventral region of barn swallows is intercorrelated, throat coloration in males and belly color in females are most strongly correlated to indicators of seasonal reproductive success (Safran and McGraw 2004), in addition to overall ventral color influencing a male’s paternity (Safran et al. 2005). Because of these patterns, we analyzed yolk compound concentrations as a function of male throat and female belly coloration.

To analyze color, we sampled patches (three to ten feathers) of ventral plumage from the throat region of males and the belly region of females and carefully mounted these colored feathers on an index card as to recreate the natural plumage appearance of the bird (Safran and McGraw 2004). These cards were stored in the dark until plumage color scoring. The color of feather samples was scored along three traditional axes of color (hue, saturation, and brightness) using a reflectance spectrophotometer (ColortronTM; Light Source, San Rafael, CA; Hill 1998); this spectrophotometer does not quantify light in the ultraviolet range, but the range in which it does quantify color is sufficient for this species because the ventral plumage of barn swallows does not exhibit a unique ultraviolet reflectance peak (Safran and

McGraw 2004). Each plumage patch was scored three times, and we averaged these scores to determine mean hue, saturation, and brightness for each region. Color scores were significantly intercorrelated within (all $p < 0.001$, all r between -0.67 and 0.82) each ventral region (throat for males, belly for females). Principal components analysis (PCA) was used to collapse hue, saturation, and brightness scores within each plumage region. The first principal component (PC1) for each region explained 81–85% of the variation in the color scores of each plumage region in both sexes. Birds with lower PC1 scores have redder (lower hue values), more saturated, and darker (lower brightness values) plumage.

Maternity and paternity certainty Although female barn swallows have been reported to occasionally lay their eggs in the nests of other females (Brown and Brown 1999), we did not detect the presence of another female's eggs in any of the nests contained in this study and have only rarely encountered this behavior in our study area. Because the shape and color patterns of barn swallow eggs are nearly unique to a female (Brown and Sherman 1989), it is easy to discern foreign eggs in a nest using visual characteristics. Therefore, we feel confident that our assignment of the social mother and genetic mother are equivalent.

Extra-pair mating is a prevalent reproductive strategy of barn swallows throughout their breeding range. As part of a separate study in our study area, nearly half of the broods we sampled (26 of 53) contained at least one extra-pair offspring. The mean proportion of extra-pair young per first brood was 0.23 ± 0.30 or equivalent to one nestling per nest (Neuman et al. 2007). The nonmonogamous genetic mating system of our study population could possibly confound the analyses of whether females allocate differentially to their eggs as a function of male quality in this study or others that were conducted in avian social systems with similar levels of extra-pair matings (e.g., many studies of egg yolk contents in European populations of barn swallows; e.g., Gil et al. 2005; Saino et al. 2002, 2003, 2006). For example, if females socially mated to males of low quality deposited greater amounts of yolk compounds, it would be difficult to discern whether these patterns were a function of compensatory mechanisms in the case that females deposit more to compensate for a poor mate, or differential allocation in the case that a female's offspring were actually sired by higher-quality extra-pair males.

Yolk androgen analyses The whole, fresh yolk was drained into a 1.5-ml Eppendorf tube, mixed with about 300 mg of distilled water, and homogenized by vortexing. The yolk was then frozen at -20 C. Androgens were extracted twice with 4 ml of petroleum ether/diethyl ether (30:70 v/v). Neutral lipids were precipitated with 90% ethanol at -20 °C.

Extracts were then transferred to diatomaceous earth microcolumns for further purification and separation of androgens following the methods of Pilz et al. (2003) and Schwabl (1993). Radioimmunoassays were conducted for androstenedione (A4), dihydrotestosterone (DHT), and testosterone (T) following the standard methods first described by Wingfield and Farmer (1975). All samples were run in a single assay for each hormone. Intra-assay variation was 7.0% for A4, 10.2% for DHT, and 8.4% for T. Recoveries averaged 64% for A4, 25% for DHT, and 51% for T, similar to other assays using this method (e.g., Pilz et al. 2003; Gwinner and Schwabl 2005). The rather low recoveries for DHT are not exceptional, as DHT recoveries are notoriously low across species, possibly by being strongly bound by yolk proteins or lipoproteins (Schwabl, unpublished results). All data used in these analyses are corrected for these recoveries. To remove the possibility of interassay variation, all samples were run in a single assay.

Yolk carotenoid analyses We analyzed yolk carotenoids via high-performance liquid chromatography (HPLC; sensu McGraw et al. 2002). We homogenized thawed egg yolks in 1 ml water and extracted lipids from 100 μ l of the homogenate with 200 μ l of both ethanol (containing canthaxanthin as an internal standard) and *tert*-butyl methyl ether. The solution was vortexed, centrifuged, and the supernatant evaporated before redissolving the residue in 200 μ l HPLC mobile phase (methanol–acetonitrile–chloroform, 46:46:8, v/v/v). We injected 50 μ l into a Waters™ 717plus Autosampler HPLC (Millipore, Milford, MA) fitted with a Develosil RPAqueous RP-30 HPLC column (250 \times 4.6 mm I.D.; Nomura Chemical, Japan), and ran an isocratic system (HP 1050 Series Isocratic Pump) of the aforementioned mobile phase for 25 min at a constant flow rate of 1.2 ml min⁻¹. We confirmed the identity of yolk pigments by comparing retention times to those for authentic reference carotenoids, including anhydrolutein, beta-carotene, and several ketocarotenoids (e.g., astaxanthin, canthaxanthin) in addition to the three we detected in swallow yolk: lutein, zeaxanthin, and β -cryptoxanthin.

Statistical analysis We applied transformations [\log or \log (compound + 1)] to normalize the distributions of all yolk compounds (both concentrations and total amounts). We used smoothing splines to graphically characterize the relationships between continuous variables; these were only used to characterize the relationship between variables of interest (e.g., whether they are linear or not) so that appropriate models could be constructed (e.g. whether higher-order terms would be necessary in linear models). Using JMP 6.0 (SAS Institute, Cary, NC), we constructed very conservative splines using a flexible tuning parameter ($\lambda = 1$) to construct pictorial relationships between variables of interest.

With the exception of analyses related to egg and yolk mass, the results of statistical comparisons are similar whether they are based on total yolk compound content (ng of androgen per whole yolk, or mg carotenoid per whole yolk) or concentration (pg androgen per mg yolk, or mg carotenoid per g yolk). Therefore, we report data for concentrations (the more commonly used measure in the literature) throughout and include results for both concentration and amount only when presenting correlations of yolk compound levels with egg-size characteristics. Note that the units listed above for androgens and carotenoids are not the same but follow the units commonly reported for these compounds in published studies (e.g., Pilz et al. 2003; Gil et al. 2005; Saino et al. 2002, 2003; McGraw et al. 2005).

Because amounts and concentrations of yolk androgens were highly intercorrelated, we used PCAs to reduce the variables representing yolk androgens. In the two separate PCAs (see details below), the first principal component explained more than 70% of the variation among the three variables entered, and we used each of these first principal components in subsequent analyses. We used PCA to reduce three androgen concentration measures into one component (Eigen value=2.13, total variance explained=71.18, with the following component loadings for A4 [0.54], DHT [0.55], and T [0.63]). A second PCA was used to reduce three androgen amount measures into one component (Eigen value=2.24, total variance explained=74.85, with the following component loadings for A4 [0.54], DHT [0.56], and T [0.62]) in egg yolk.

We separated our measures of concentration and total amounts of yolk androgens because we a priori do not have predictions for whether either measure is more biologically meaningful. For both PCAs, all component loadings are positive and as such, higher PC1's indicate higher concentrations/amounts of yolk androgens, and as such, results for each androgen separately follow the same patterns as the results we report for the variables derived from PCA.

It is interest to note that the carotenoid variables we measured were not highly intercorrelated (see “Results”), and instead of reporting the results of each one separately, we report a composite score (total carotenoid content) to indicate the total amounts and concentrations of the three carotenoids we measured in this study. We did this because we had no a priori expectation that the three types of carotenoids in barn swallow eggs would differentially relate to our indicators of maternal and paternal quality.

Using PROC MIXED (SAS version 8.1), we applied generalized mixed linear models to quantitatively test the predictions of the Investment Hypothesis outlined in Table 1. Unless noted, the lack of independence of eggs and variation because of variation within a clutch and a given female, in addition to laying order effects, was controlled for by using “nest” and “laying order” as the

random effects in the model. When significant at $\alpha < 0.05$, random effects are listed in italics in the “Results” section, along with the test statistics for fixed effects. We used the Satterthwaite method to estimate the denominator degrees of freedom, which performs well with unbalanced data (Littell et al. 2002) or, in our case, unequal clutch sizes.

Because the egg collection was mostly conducted at new breeding sites in our study area, vs sites where we have longer-term data sets with known-age individuals, we do not have accurate data on individual ages for this data set. Thus, we do not report relationships between yolk compounds and male and female age here.

Results

Correlation between yolk carotenoid and androgen concentrations

Concentrations of lutein and zeaxanthin were positively correlated in egg yolks ($F_{1, 93}=45.33$, $P < 0.0001$; *nest, laying order*), as were concentrations of β -cryptoxanthin and zeaxanthin ($F_{1, 98.2}=95.25$, $P < 0.0001$; *nest, laying order*). Concentrations of lutein and β -cryptoxanthin were not correlated with one another ($F_{1, 92.2}=0.51$, $P=0.47$; *nest*). Concentrations of all three androgens were positively correlated with one another (all F between 22.17 and 41.41, all $P < 0.0001$; *nest* is a significant random effect in all models). When considered singly, concentrations of none of the androgens were significantly correlated with any of the carotenoid concentrations (all F between 0.02 and 1.52, all $P > 0.22$; *nest* is a significant random effect in all models).

Tests of the investment hypothesis

Variation among females and within clutches: a prerequisite of the investment hypothesis

Interfemale variation. Using “nest” as a fixed effect in our model, we found that egg weight (g), yolk weight (g), and the total concentrations of yolk carotenoids and androgens were significantly variable among females (effect of “nest”: all $F_{23, 110}$ between 5.36 and 16.45, all $P < 0.0001$; *laying order* significant for egg and yolk weight and for carotenoid variables).

Intrafemale variation/laying order effects. Egg weight increased with lay order (Fig. 1a), whereas we found no relationship between yolk weight and lay order (Fig. 1b). Carotenoid concentration decreased with lay order (Fig. 1c), whereas androgen concentrations did not vary with lay order (Fig. 1d).

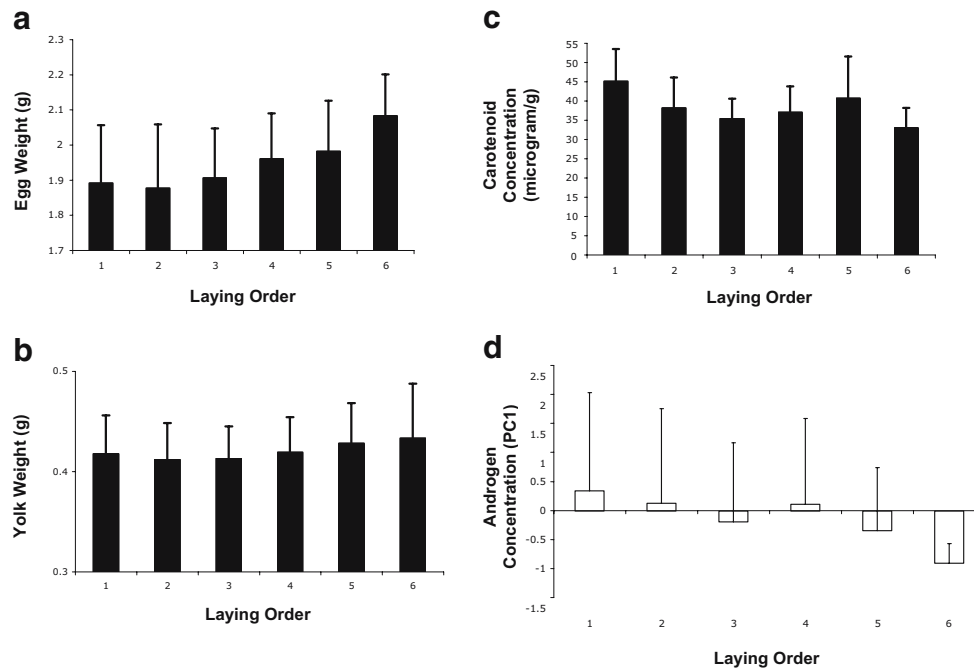


Fig. 1 **a** Egg weight ($F_{23, 110}=24.94$, $P<0.0001$ nest) but not **b** yolk weight ($F_{23, 110}=1.92$, $P=0.16$, nest) increased with laying order. **c** Total carotenoid concentrations decreased with laying order ($F_{23, 110}=6.18$, $P=0.02$ nest). **d** Androgen concentrations did not vary with laying order

($F_{23, 110}=2.15$, $P=0.14$ nest). Variables in *italics* indicate the significance of a random effect in a mixed model where “laying order” was added as a fixed effect; least square mean \pm SD are portrayed in all figures, $N=23$ clutches

Egg and yolk mass

Heavier eggs contained heavier yolks (yolk mass $F_{1, 110}=78.29$, $P<0.0001$; mass $F_{1, 110}=0.23$, $P=0.62$ laying order, nest). Although egg mass and yolk mass are highly correlated, we found some differences in the concentrations and amounts of yolk compounds when we analyzed them as a function of either egg or yolk weight (Table 2). For

example, heavier eggs (egg mass) had lower concentrations of carotenoids, whereas we found no such relationship between this yolk compound and yolk mass. Furthermore, heavier yolks had greater total amounts of carotenoids, whereas we found no relationship between egg mass and the total amounts of carotenoids. The patterns for androgens are much more consistent: We found no relationship between androgen concentrations as a function of either egg or yolk

Table 2 Concentrations and amounts of yolk compounds as a function of egg and yolk mass

Variable	Sign of relationship	Egg mass (g)	Sign of relationship	Yolk mass (g)
Carotenoid concentration	–	$F=331.21$ $P<0.0001$ <i>N</i>		$F=3.13$ $P=0.80$ <i>N, LO</i>
Total amount carotenoids		$F=0.78$ $P=0.37$ <i>N</i>	+	$F=6.19$ $P=0.01$ <i>N, LO</i>
Androgen concentration		$F=1.68$ $P=0.20$ <i>N</i>		$F=1.03$ $P=0.31$ <i>N</i>
Total amount androgens	+	$F=3.14$ $P=0.03$ <i>N, LO</i>	+	$F=6.90$ $P=0.009$ <i>N</i>

Results are based on Mixed Linear Models where both “nest” and “laying order” are controlled for as random effects. The statistical significance of random variables are indicated for each analysis in *italics* as *N* for nest and *LO* for laying order. When significant, the nature of the relationship is indicated as – for negative and + for positive. The sample size=23 clutches for all analyses.

mass, but larger eggs and yolks had greater total amounts of androgens.

Clutch size

Carotenoid (total carotenoid concentration: $F_{1, 82}=0.37$, $P=0.54$; laying order) and androgen (PC1 androgen concentration, $F_{1, 82}=0.12$, $P=0.67$) concentrations did not vary with clutch size.

Timing of breeding

Early-breeding females deposited lower concentrations of carotenoids into egg yolk (total carotenoid concentration: $F_{1, 82}=7.17$, $P<0.01$; laying order; Fig. 2). Yolk androgen concentration did not vary with lay date (PC1 androgen concentration: $F_{1, 82}=0.34$, $P=0.55$).

Yolk compounds and maternal phenotype

More colorful females with longer tail streamers deposited lower concentrations of carotenoids into their egg yolks compared to less colorful females with shorter streamers (belly color: $F_{1, 77}=6.58$, $P=0.02$; Fig. 3a; tail streamer length: $F_{1, 77}=3.85$, $P=0.05$ laying order; Fig. 3b). We found no effect of either female plumage color or streamer length on yolk androgen concentration (both $F<0.14$, both $P>0.70$; no significant random effects; Fig. 3c,d).

Yolk compound deposition and phenotype of social father

Neither the streamer length nor throat color of a female's social mate affected carotenoid levels in egg yolks (both $F<1.15$, both $P>0.30$; Fig. 4a,b), whereas females mated to more colorful males laid eggs with greater concentrations of yolk androgens into their eggs (PC1 male throat color: $F_{1, 81}=6.02$, $P=0.02$; Fig. 4c; male streamer length $F_{1, 81}=0.46$, $P=0.50$; Fig. 4d).

Discussion

Correlated deposition of carotenoids and androgens?

Correcting for lay order effects, we found no correlation between yolk androgen and carotenoid levels in individual barn swallow eggs. Two previous studies of yolk carotenoids and androgens in barn swallows did not test for such a correlation; however, it is reasonable to suspect a similar pattern in those studies because they, like us, found discordant relationships between each compound and laying order (Saino et al. 2002; Gil et al. 2005). As such, it appears that patterns of carotenoid and androgen

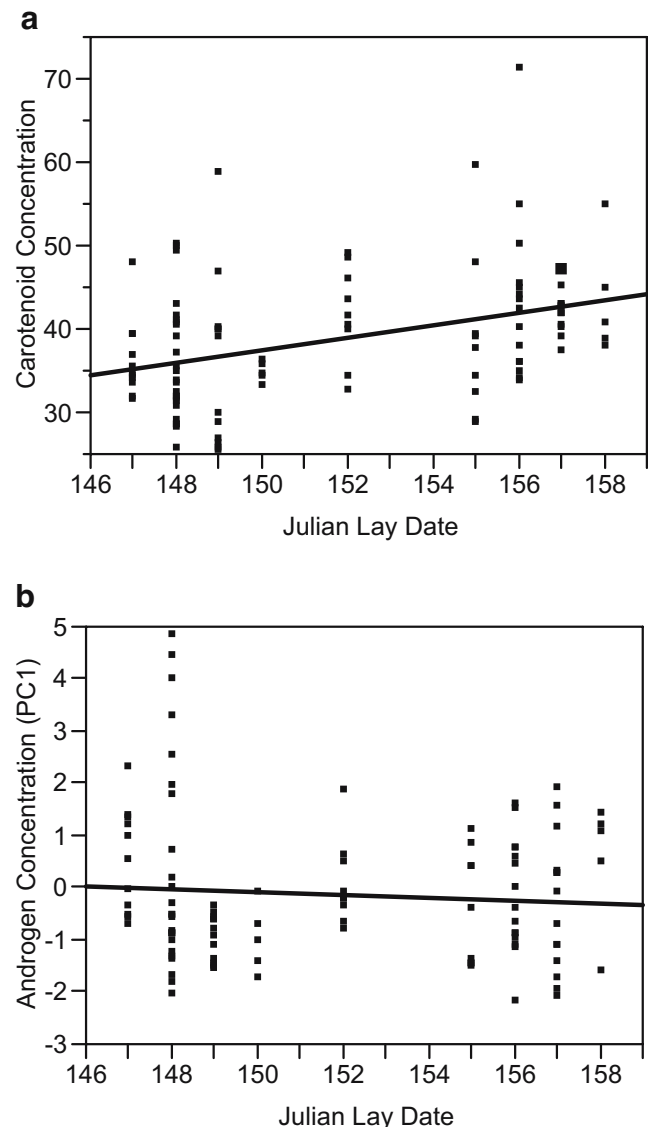
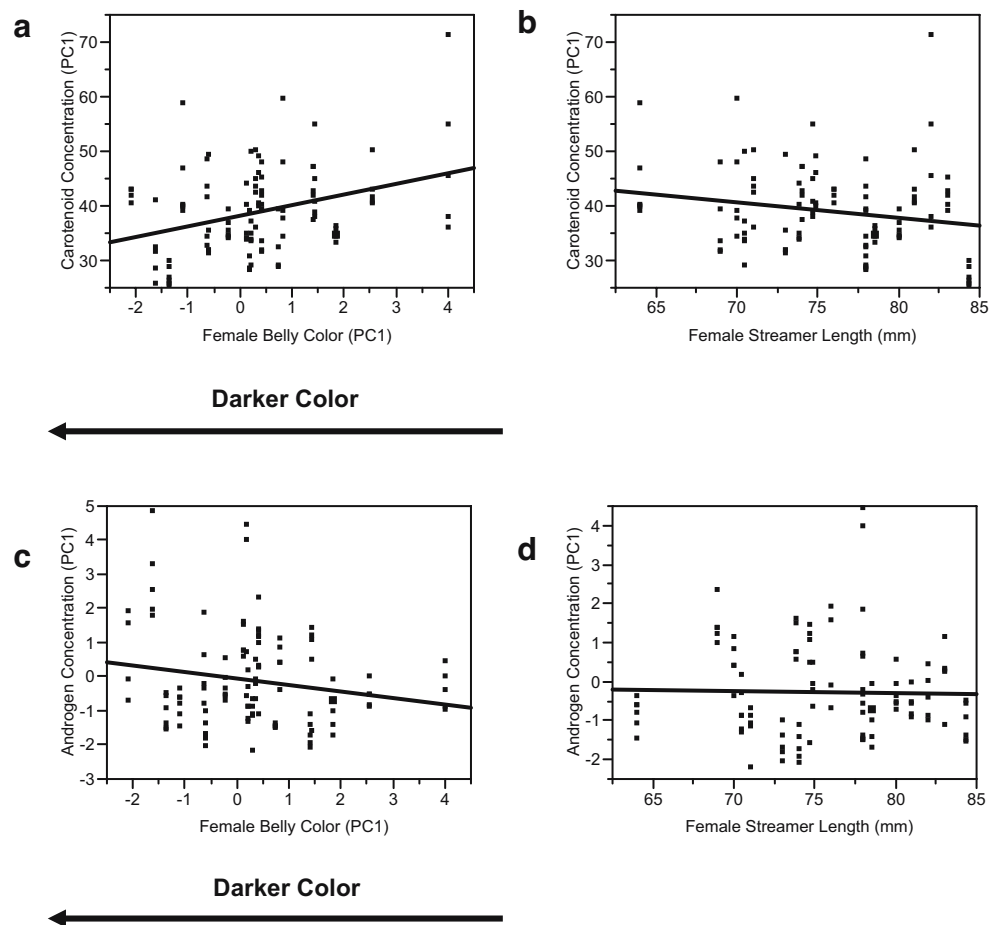


Fig. 2 **a** Average concentrations of yolk carotenoids per clutch increased with Julian lay date, whereas **b** average concentrations of yolk androgens per clutch do not vary significantly with Julian lay date. $N=23$ clutches. Julian lay date is a transformed calendar date where January 1=Julian day 1. $N=23$ clutches

deposition in barn swallows differ from those in two populations of lesser black-backed gulls, where it was found that first-laid eggs contained greater amounts of carotenoids but lower concentrations of androgens than second- and third-laid eggs (Royle et al. 2001; Verboven et al. 2005) and also from the positive associations found between these yolk compounds in house finches (Navara et al. 2006). Differences between these species in their social environment (e.g., extent of coloniality) or other life-history parameters (e.g., clutch size, number of clutches produced per season, lifespan) may affect allocation patterns of these yolk compounds within and among eggs (Pilz and Smith 2004; Verboven et al. 2005). For example, intraspecific interactions within colonies has been shown to cause

Fig. 3 Concentrations of yolk carotenoids decreased as a function of **a** female belly color and **b** female streamer length, whereas concentrations of yolk androgens did not vary as a function of **c** female color or **d** streamer length. In **a** and **c**, lower PC scores indicate darker birds. $N=21$ clutches



differences in the amounts of yolk compounds in the eggs of lesser-black backed gulls (Verboven et al. 2005), whereas group size in the facultatively colonial barn swallow is unrelated to yolk carotenoid or androgen levels (Gil et al. 2005; Pilz et al., unpublished data).

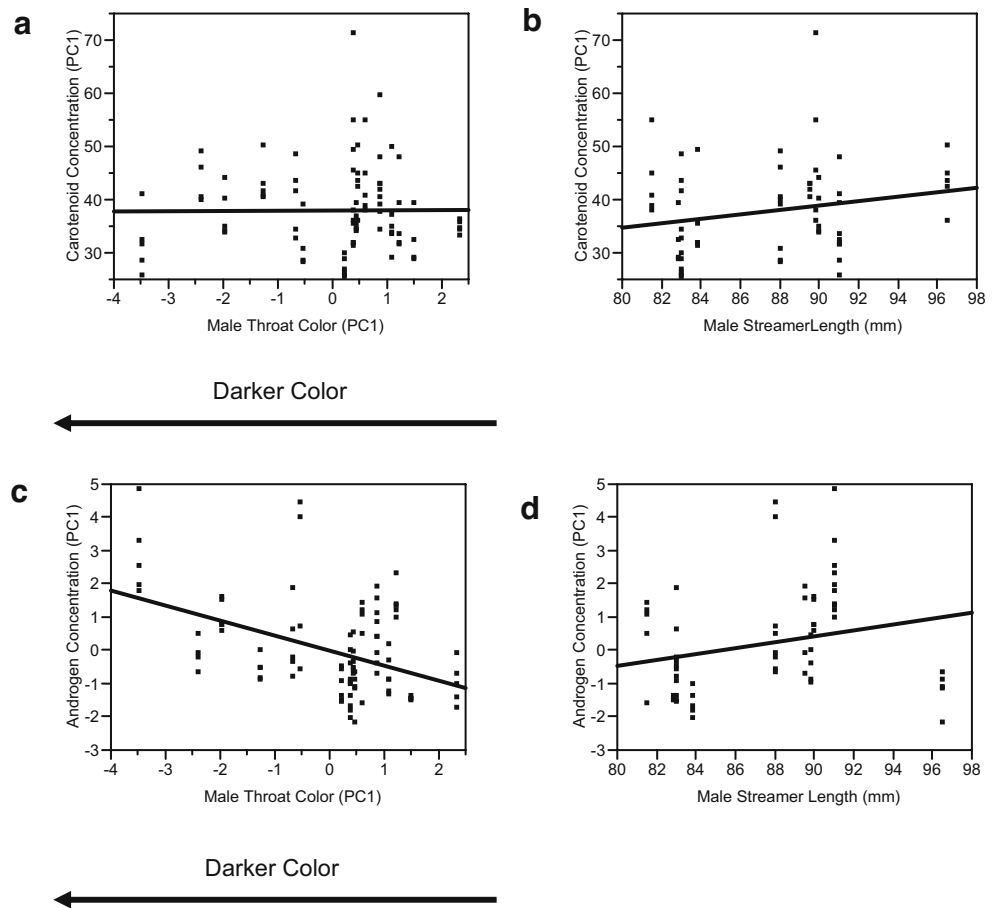
Why might there be no association between yolk carotenoids and androgens in barn swallow eggs? At the mechanistic level, the transmission of these compounds into egg yolk may be regulated by different processes. For example, carotenoid levels declined with laying order in our study (as in many others; Royle et al. 1999, 2003; Blount et al. 2002; Saino et al. 2002, 2003), whereas androgen levels did not vary with laying order. Carotenoids partitioned into yolk are lost from the female body, and this loss might exceed the gain from diet (Bortolotti et al. 2003). Androgens, in contrast, are synthesized and are not subject to such direct dietary constraints (although diet can influence androgen allocation in zebra finches, *Taeniopygia guttata*; Rutstein et al. 2005). At the functional level, offspring may differentially benefit from varying amounts of these compounds, depending on their genetic quality/diversity, parasite exposure, hatching order, and other phenotypic conditions. Thus, “more” may not be “better” for all individuals, and future studies should carefully

consider such optimization schemes that can be overlooked by examining coarse correlations between carotenoids and androgens across all individuals.

Support for the investment hypothesis?

The investment theory predicts patterns of differential allocation from parents to offspring such that high-quality parents provide more resources than parents of lower quality. If yolk compounds including carotenoids and androgens of maternal origin represent a form of parental investment, then according to the Investment Hypothesis (Gil et al. 1999; Pilz et al. 2003), only high-quality females are able to allocate greater amounts of these yolk compounds to benefit their offspring. Our results for the deposition of yolk carotenoids, especially when statistically significant patterns emerged, consistently contradicted the predictions of the Investment Hypothesis. The most colorful female barn swallows that bred earliest in the year and laid heavier eggs—both well-established predictors of seasonal reproductive success in birds (Drent and Daan 1980, van Noordwijk and de Jong 1986; Price 1998; Safran 2004; Ardia 2005), including barn swallows—deposited lower concentrations of yolk carotenoids. Saino et al.

Fig. 4 Concentrations of yolk carotenoids did not vary as a function of **a** male throat color or **b** streamer length. Females paired to darker males deposited **(c)** greater amounts of androgens to eggs. **d** Male streamer length did not affect the allocation of androgens to the eggs of his social mate. In **a** and **c**, lower PC scores indicate darker birds. $N=21$ clutches



(2002) also reported that female barn swallows deposited lower concentrations of carotenoids in the eggs fathered by high-quality males. One potential reason for such results is that dietary carotenoid availability may increase seasonally and carotenoids may therefore increase in eggs laid later in the season. Barn swallows begin breeding when local deciduous flora has just finished leafing and is not yet at its fullest level of primary productivity, when peak carotenoid abundance (as accessory photosynthetic pigments in plants) is expected in their herbivorous insect prey. Alternatively, birds returning earliest from migration may have depleted more of their carotenoid stores (e.g., because of increased energy investment or decreased food intake en route), leaving them with fewer amounts to allocate to egg yolk. Still, there is substantial variation in yolk carotenoid levels among birds laying eggs on the same day (e.g., on 30 May, which is close to the modal clutch initiation date in our study, the mean \pm SD of total carotenoid levels = 33.86 ± 6.47 mg/g yolk, $n=10$ eggs), indicating that seasonal effects themselves cannot fully account for all observed variability in swallow yolk carotenoid levels.

It is interesting to note that although not consistent for all predictors of seasonal reproductive success, we found some patterns of androgen deposition that are consistent with

predictions of the Investment Hypothesis. Although we found no effect of laying order on the deposition of yolk androgens, our data indicate that heavier eggs and yolks contained greater total amounts of androgens compared to smaller eggs and that females deposited more androgens in eggs when paired to a more colorful male. The effect of male color on the deposition of androgens is also consistent with the differential allocation hypothesis (Burley 1988), which predicts that females invest more in their young when paired with a high-quality mate; such a pattern is likely not confounded by the nonmonogamous mating system of barn swallows because females paired to dark males are less likely to pursue extra-pair mates (Safran et al. 2005). Taken together, these results provide some, although not overwhelming, evidence that the deposition of androgens to egg yolks is adaptive, although these results remain to be tested experimentally, and the actual costs and consequences of androgen deposition await further study before this conclusion is considered robust.

Despite the limitation of a correlational data set, our results nevertheless partially correspond to a similar study of barn swallows in Europe, where it was found that some patterns of yolk androgen deposition appears to be adaptive (Gil et al. 2005). Like our findings, this study also

demonstrated that androgen concentrations did not vary with laying order and that indicators of both male and female quality predicted the concentrations of at least one yolk steroid, A4, in egg yolks, although one notable but perhaps trivial difference between this study and ours is that Gil et al. (2005) extracted only a portion of the yolk, whereas our analyses are based on the entire yolk. Specifically, Gil et al. (2005) found that early-arriving females with greater clutches deposited greater concentrations of this yolk androgen into their eggs compared to females arriving later to breeding grounds. Using tail-streamer manipulations, this study also reported that females paired with longer-streamered males deposited more A4 into their eggs (Gil et al. 2005). However, counter to the results of Gil et al. (2005), Saino et al. (2006) found no relationship between A4 deposition and male streamer length in a similar experimental setup. While we did not analyze the deposition of yolk compounds as a function of arrival date to breeding sites as did Gil et al. (2005), we did not detect a significant relationship between yolk androgen concentrations and Julian laying date, a good proxy of arrival date (Safran, unpublished data) and an important predictor of reproductive success in our study area (Safran 2004; Safran and McGraw 2004). Despite the fact that our conclusions are not based on experimental data, we still detected a relationship between concentrations of yolk androgens and male coloration, a sexually selected trait in our population of barn swallows (Safran and McGraw 2004; Safran et al. 2005). That we found a clear relationship between yolk androgen concentration and color but not male streamer length—an interesting contrast to the findings of Gil et al. (2005) but similar to those of Saino et al. (2006)—corroborates our other findings that male coloration but not the length of his streamers appears to be under stronger sexual selection than streamer lengths in our study area (Safran and McGraw 2004; Safran et al. 2005).

Total or relative amounts of androgens and carotenoids—does it matter?

Similar to Pilz et al. (2003), our results differed depending on whether we considered the concentrations or total amounts of yolk compounds. For example, accounting statistically for laying order and nest effects, we found that heavier eggs contained lower concentrations of carotenoids but detected no relationship between egg weight and the total amount of carotenoids in yolks.

To shed light on whether underlying mechanisms that produce differences in total vs relative amounts of yolk compounds are biologically different, it will be important to discern whether females are actively vs passively depositing these molecules to their offspring. Because the two different measures of yolk compounds seem to produce

varying results in at least two studies, we suggest that studies also report both the relative and total amounts of yolk compounds in eggs so that data on this interesting pattern continue to accumulate (e.g., McGraw 2006). Another reason studies should report both total amounts and concentrations of yolk compounds is that we do not understand which of these measures is biologically more meaningful in terms of exposure of the developing embryo to these compounds. For example, a higher concentration may cause a higher dose of a compound during a given interval of embryonic development, whereas a greater amount may mean prolonged exposure during development. Discerning the difference between these two possible strategies will await an understanding of the temporal pattern of androgen receptors in the developing embryo.

Understanding the mechanisms by which compounds are transmitted into the yolk (e.g., actively or passively) would also illuminate how high-quality females differ from low-quality females, thereby allowing researchers to discern more precisely the predictions of the Investment Hypothesis and a possible alternative explanation, the Epiphenomenon Hypothesis, which predicts that yolk biochemicals are variably but passively transferred to offspring as an epiphenomenon of maternal physiology (Schwabl 1993; Bortolotti et al. 2003). For example, high-quality females may deposit greater amounts of yolk compounds than low-quality females simply because there are more of these compounds in circulation (passive, not active deposition and evidence for the Epiphenomenon Hypothesis). Alternatively, high-quality females could be actively depositing these compounds to offspring, perhaps at the cost of diminishing the availability of these compounds to themselves (active deposition and evidence for the Investment Hypothesis). Comparisons of populations in different environments, such as those reported by Badyaev et al. (2006), also provides some insights into mechanisms of deposition.

Summary

We found no correspondence both within eggs and among females between the deposition of androgens and carotenoids to egg yolks, suggesting that the deposition patterns of each compound should be considered separately and may also be mediated by and responsive to different physiological and environmental factors. Although we did find evidence that the deposition of carotenoids to egg yolks is nonrandom, the significant patterns we report in this paper counter the predictions of the Investment Hypothesis. Interestingly and worth further investigation, we detected a pattern that might represent an adaptive tradeoff in deposition of resources between mother and

offspring: Heavier eggs had lower concentrations of carotenoids, although heavier yolks had great total amounts of carotenoids. Although the relationship between egg size and offspring fitness is not straightforward (Williams 2005), it is believed that both yolk carotenoids and other materials deposited in eggs are costly to allocate. As such, females may be solving the problem of not being able to simultaneously allocate all important resources by trading off among them.

As in other populations of barn swallows (Gil et al. 2005), we detected some patterns that are consistent with adaptive patterns of androgen deposition, as predicted by the Investment Hypothesis.

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