Female collared flycatchers adjust yolk testosterone to male age, but not to attractiveness

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The differential allocation hypothesis predicts that females invest more resources into reproduction when mating with attractive males. In oviparous animals this can include prefertilization decisions such as the production of larger eggs and the deposition of hormones, such as the steroid testosterone, into yolks. On the other hand, a compensatory hypothesis posits that females allocate more resources into the eggs when mated with males of inferior quality. In the present study, we show that free-living females of the collared flycatcher (*Ficedula albicollis*), a small passerine bird, do not produce larger eggs or deposit more testosterone into eggs when mating with attractive males reflected by a large forehead patch size, which is contrary to the prediction of the differential allocation hypothesis. However, we found higher yolk testosterone concentrations in eggs laid for young than older males. Because in young males genetic quality, parental experience, or willingness to invest into paternal care is likely to be low, high yolk testosterone level in their clutches may indicate that their females follow a compensatory tactic. They may elicit more paternal care from young, inexperienced males by hormonally increasing nestling begging. Laying date was also correlated with yolk testosterone level; however, when we controlled for it, male age still remained a strong determinant of testosterone allocation. *Key words:* differential maternal investment; sexual selection; yolk testosterone; wild bird population. *[Behav Ecol 16:383–388 (2005)]*

Good genes models of female mate choice posit that females obtain viability genes for their offspring by mating with males that express attractive phenotypic traits (Andersson, 1994). Experimental tests of the good genes hypothesis of female choice are hampered by the differential allocation hypothesis, which posits that females increase their investment into reproduction when mated to attractive males (Burley, 1988; Sheldon, 2000; Williams, 1966). In birds, this differential allocation comprises variation in postnatal offspring care (Burley, 1988) and prefertilization decisions such as the number of eggs laid (Petrie and Williams, 1993), egg size (Cunningham and Russell, 2000), sex ratio (Ellegren et al., 1996), and possibly the deposition of biologically active molecules, such as the steroid testosterone, into egg yolk (Gil et al., 1999, 2003) that can enhance chicks' begging vigor, competitive ability, and growth rate (Eising et al., 2001; Schwabl, 1993, 1996). However, an adverse effect of testosterone was also demonstrated, as the mortality rate of chicks that hatched of testosterone-injected eggs was higher (Sockman and Schwabl, 2000). In barn swallows (*Hirundo rustica*), females deposited more antiviral antibody when mated to attractive males with long tails, which also supports the validity of the differential allocation hypothesis (Saino et al., 2002b). An alternative to the allocation hypothesis is that females allocate more to reproduction if their mate is unattractive and/or low quality, for example, when he is young and inexperienced. Such a compensatory mechanism was found in barn swallows, in which females transferred more carot-

enoids into the yolk when they had unattractive partners (Saino et al., 2002a). Compensation may apply to androgens as well. If females allocated more testosterone to clutches laid for young or inexperienced males, the predicted stronger begging activity (Schwabl, 1996) could elicit more nestling care in these males.

Studies in support of the good genes models, for example, one in the collared flycatcher (*Ficedula albicollis*; Sheldon et al., 1997) in which broods with mixed paternity were used, have controlled for postnatal differential allocation, but they have not accounted for prefertilization allocation or resources and hormones to the eggs. If adjustments of egg quality and yolk hormones occur under conditions in which females are free to choose among males of variable attractiveness and presumably genetic quality, one is faced with the possibility that evidence for the good-male-genes hypothesis of mate choice found in previous studies, including one of the collared flycatcher (Sheldon et al., 1997), was based on maternal effects rather than on male genetic variation.

In species with paternal care, female resource allocation may be sensitive to the quality of care the male will provide for the offspring, which likely varies with his experience (Forslund and Pärt, 1995) and willingness to invest into the current reproductive event (Clutton-Brock, 1991) and hence his age. Moreover, older males are likely to be of higher genetic quality (Brooks and Kemp, 2001) although this may depend on the trade-off between mating effort and survival. Hence, females mating with such males may either obtain direct fitness benefits, if older males have territories of high quality (Alatalo et al., 1986a), provide superior parental care through improved foraging or defense against predators (Alatalo et al., 1986b; Marchetti and Price, 1989; Yasukawa, 1981) or pathogens (Saino et al., 2003), or provide genetic

benefits, if viability is heritable (Brooks and Kemp, 2001; Trivers, 1972). As a result, females would benefit from differential allocation depending on the age of their mate.

In the present study, we investigated the role of male quality reflected by age and the expression of a sexually selected plumage trait, the size of the forehead patch, on differential allocation of testosterone into the eggs by female collared flycatcher. The collared flycatcher provided an ideal system to test predictions of both the differential allocation and the compensatory hypotheses in a wild bird population, in which females are free to choose among males of different pheno-types. Male collared flycatchers exhibit a secondary sexual trait, the conspicuous white forehead patch (Gustafsson et al., 1995), which varies in size (Hegyi et al., 2002). Females prefer males with a large forehead patch, especially as extra-pair mates (Michl et al., 2002; Sheldon and Ellegren, 1999). The differential allocation hypothesis predicts that the egg investment (egg size, yolk size, testosterone concentration) of a collared flycatcher female is higher if her mate has a large forehead patch or is an old and experienced male. The predic-tion of the compensatory model is that females allocate more resources into the yolk when mated to a male with a small forehead patch or to a young male.

Fledglings sired by males with large forehead patches are in better condition than are those whose fathers have small forehead patches (Sheldon et al., 1997). This is interpreted as evidence for the good genes hypothesis of female choice and sexual selection. However, maternal effects through hormone deposition or nutrient allocation in response to male attractive-ness could have contributed to or mimicked these good gene effects.

METHODS

We worked on an artificial nest-box plot in the Pilis Mountains, Hungary (47°42′ N, 19°01′ E), in a Central European oak forest, a typical habitat for the collared flycatcher. We trapped males during the egg-laying period and measured the height and width of their white forehead patch to the nearest 0.1 mm. We calculated the size of the forehead patch as the product of maximum width and height. There is a difference in the plumage of 1-year-old and adult collared flycatcher males; therefore, we distinguished them from each other based on criteria in Svensson (1992). The coloration of 2-year-old and older males is very similar, so these age groups cannot be categorized any further.

Female quality was characterized by three morphological traits. Tarsus length and wing length were measured with a caliper to the nearest 0.1 mm. Body mass was measured with a Pesola spring balance to the nearest 0.1 g. As the age of females is difficult to identify on the basis of morphological traits, we only used age data of nine females that were ringed as fledglings. The timing of breeding can influence female reproductive success (Garamszegi et al., 2004b) in this species. Therefore, we included the laying date (related to the median laying date in the population in the focal year) in all analyses to control the seasonal variation in androgen deposition.

Yolk testosterone assay

We collected eggs of randomly chosen clutches on the day they were laid, replaced them with dummy eggs, and stored them at 5°C. When a clutch was complete, we took samples from the yolks of six-egg clutches ($N = 25$) immediately and stored them at $-20°C$ until we measured testosterone con-centrations. Clutches, therefore, were stored at 5°C for the same duration.

Yolk testosterone was determined by RIA (Adkins-Regan et al., 1990). We homogenized 2–10 mg of yolk with 200 µl distilled water and 2000 c.p.m. of H^3-testosterone (for recovery) at room temperature, after which the mixture was gently stirred and left to settle for 1 h. We added 500 µl 1% sodium-dodecil-sulphate (SDS) and incubated the mixture for 0.5 h at 37°C. SDS is an effective emulsifier that "opens up" the lipid-coated yolk particles and results in the decrease of interfering substances, thereby increasing the precision of the testosterone RIA (Kelemen et al., 2003). The mixture was left to settle for some minutes at room temperature and was then extracted three times with diethyl ether. We dried the combined extracts and redissolved them in absolute ethanol. After overnight delipidization at $-20°C$, we washed the ethanol phase twice with petroleum ether and re-extracted the petroleum ether phases with absolute ethanol. The combined ethanol phases were dried and resuspended in assay buffer. Testosterone was quantified in duplicates of three different dilutions for each sample in a single assay. The samples were randomly distributed across assay. Recoveries ranged from 60–75%, and intra- and interassay coefficients of variation were 8–12% and 9–15%, respectively. Sensitivity of the assay was 5 pg per tube. The testosterone antibody (Edquist; final dilution of 1:8000 and 45% total binding) has the following cross-reactivity: 5α-dihydrotestosterone, 62%; androstenedione, 12% (Péczely et al., 1980).

Egg and yolk volume

We measured length (L) and width (W) of each egg with a caliper and calculated egg volume using the formula $V = 0.507 \times L \times W^2$ (Hoyt, 1979). We measured yolk diameter and calculated yolk volume as that of a sphere.

Statistical analysis

We applied ANCOVA for repeated measures to determine whether laying order, male attractiveness, and age affected yolk androgen concentrations and yolk and egg size. The laying date was included as a covariate in all analyses. We used general linear models with backward stepwise model selection to investigate if mean clutch yolk androgen concentrations and gamete size were related to the sizes of the forehead patch (attractiveness) within male age classes. We applied multiple stepwise regression to obtain the relationships between mean clutch testosterone investment and female morphological characteristics (body mass, tarsus and wing length). We used linear regression to analyze the association between female age and yolk testosterone levels. Instead of analyzing residuals of body mass on tarsus length, we used the standard multiple regression model that yields unbiased parameter estimates (Freckleton, 2002). We present concen-trations of testosterone (pg/mg wet yolk) as a dependent variable, but results are similar when contents (ng/yolk) are considered. As testosterone levels were normally distributed across samples, we did not apply any data transformation. All computations were run with the SPSS for Windows program package (version 10.0, SPSS Inc. 1999).

RESULTS

Yolk testosterone concentrations did not vary with the laying order of the eggs in a clutch (Table 1). The within-clutch pat-tern was not correlated with the size of the male forehead patch (attractiveness) or age (Figure 1 and Table 1). Also, the size of the white forehead patch of the male had no influence on mean yolk testosterone concentrations within male age classes, as shown by the nonsignificant age–forehead interaction in

Table 1

Results of repeated-measures ANCOVA of egg and yolk volume and yolk testosterone concentrations (T) in relation to male age (main factor), male attractiveness (size of the white forehead patch, covariate), laying date (covariate) and laying order in a clutch (repeated measures factor)

	Egg volume (mm^3)			Yolk volume (mm^3)			T (pg/mg)		
	df	F	p	df	F	p	df	F	p
Age	1(21)	0.013	.911	1(21)	1.818	.192	1(21)	15.899	.001
Forehead patch size	1(21)	1.057	.316	1(21)	0.508	.484	1(21)	0.321	.577
Laying date	1(21)	2.236	.150	1(21)	0.001	.979	1(21)	7.411	.013
Laying order	5(105)	0.689	.633	5(105)	0.787	.561	5(105)	1.207	.311
Age × laying order	5(105)	1.178	.325	5(105)	1.002	.420	5(105)	0.761	.580
Forehead × laying order	5(105)	0.483	.788	5(105)	0.878	.499	5(105)	1.331	.257
Laying date × laying order	5(105)	0.349	.882	5(105)	0.789	.560	5(105)	0.833	.529

general linear model analyses (Figure 2 and Table 2). However, females mated to young males laid eggs with higher yolk testosterone concentrations than did females mated to older males (Figure 1 and Table 1). There was a significant positive association between laying date and testosterone concentration (Table 1). Neither yolk nor egg volume was related to egg order or laying date (Table 1). Similarly, clutch means of yolk and egg volume showed no connection with male age and male attractiveness (Figures 3 and 4 and Table 2). Yolk testosterone concentrations were not related to female phenotype (tarsus length, body mass, and wing length: multiple stepwise regression: $r^2 = .032$, $F = 0.101$, $p = .905$, df = 2,6) and female age (nine females whose known ages ranged from 1 to 4 years: linear regression: $r = -.627$, $F = 3.882$, $p = .096$, power = 0.374).

DISCUSSION

We found no support for differential allocation by females in relation to mate attractiveness in the present field study in which female collared flycatchers could freely choose among males of many of different phenotypes. Females did not deposit more androgen into their eggs, nor did they produce larger egg yolks or eggs when mated to males that exhibit an attractive plumage signal. This contrasts with two laboratory studies in which zebra finches, *Taeniopygia guttata*, (Gil et al., 1999) when force-mated to presumably attractive males and canaries, *Serinus canaria* (Gil et al., 2003), when hearing preferred songs deposited more yolk androgens into their eggs. These studies had challenged the previous experimental evidence for the good genes hypothesis of female mate choice by suggesting that female prefertilization decisions such as differential hormone or nutrient allocation to eggs could have mimicked or contributed to good genes effects on offspring quality. In a previous study with collared flycatchers in Sweden, the forehead patch size of pair and extrapair partners and the fledgling body mass of their genetic offspring were compared in broods of mixed paternity (Sheldon et al., 1997). The results showed that the forehead patch size of extrapair males and the body mass of their offspring were larger than those of pair males. This experiment had controlled for posthatching but not for prefertilization maternal effects (Sheldon et al., 1997). Now the present study renders maternal hormonal or egg size effects unlikely. Therefore, it is likely that a sexually selected trait can honestly signal the genetic quality of a male and that genes inherited from the father influence nestling condition at least in the collared fly catcher.
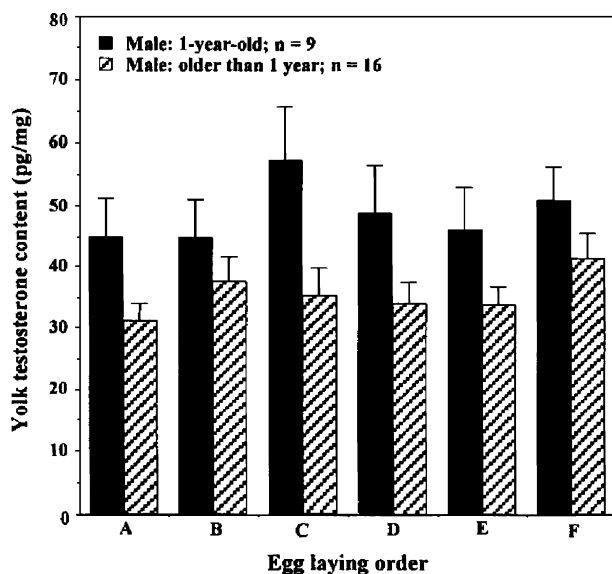

Figure 1
Mean testosterone concentrations (with SEs) in the eggs of six-egg clutches, the modal clutch size, of collared flycatchers in relation to male age.
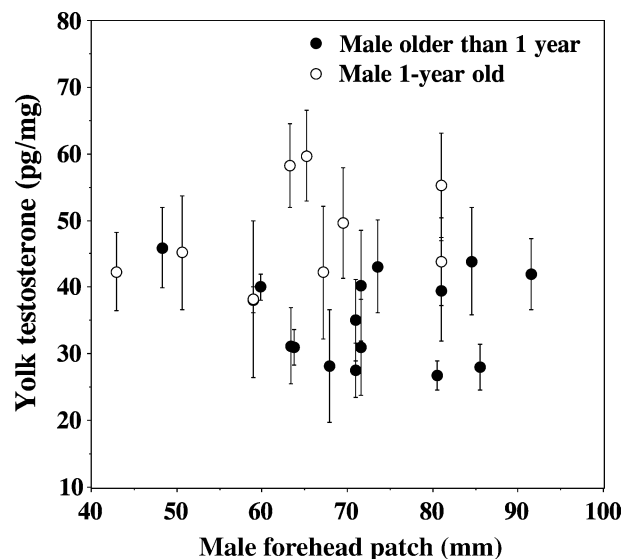

Figure 2
Yolk testosterone concentrations (clutch means with SEs) in relation to male attractiveness, the size of the male's white forehead patch, and male age.

Table 2

Results of general linear models for clutch means (n = 25) of egg and yolk volume and yolk testosterone concentrations (T) as dependent variables in relation to male age (factor), male forehead patch size (covariate) and laying date (covariate)

	Egg volume (mm^3)		Yolk volume (mm^3)		T (pg/mg)	
	F	p	F	p	F	p
Age	0.017	.898	3.188	.087	19.909	.001
Forehead patch size	1.308	.265	0.412	.247	0.321	.577
Laying date	2.622	.119	0.233	.634	7.355	.013
Age × forehead patch size	0.490	.492	1.002	.420	0.263	.613
Age × median laying date	0.771	.390	1.755	.200	0.098	.758

Our results also bear on the elusive mechanism of facultative sex ratio adjustments in birds. A previous study showed that the proportion of sons in broods of the collared flycatcher in Sweden increases with the attractiveness of the male (Ellegren et al., 1996). We found no correlation of yolk testosterone concentrations and the attractiveness of the male. Therefore, ovarian or yolk testosterone levels alone are unlikely to be part of a proposed hormonal mechanism of adjustments of primary sex ratio (Petrie et al., 2001) in the collared flycatcher. It is to be noted here that conclusions concerning good gene effects and sex ratio adjustment are only valid if yolk testosterone allocation follows a similar pattern in our and the Swedish populations.

There was no systematic variation of yolk testosterone concentrations with the order of laying of eggs of collared flycatchers. Such an egalitarian within-clutch testosterone allocation is consistent with synchronous egg hatching and similar egg mass across laying order. It suggests that, contrary to many other species in which yolk androgens vary systematically with laying order (Eising et al., 2001; Schwabl, 1993; Schwabl et al., 1997; Sockman and Schwabl, 2000), female collared flycatchers do not hormonally favor or handicap any particular young depending on the laying order in their broods, and pursue a brood survival instead of a brood reduction strategy (Cichoń, 1997).

Although females did not respond to male attractiveness with adjustments of yolk testosterone concentrations or gamete size, they seemed to vary yolk testosterone concentrations with male age. Yolk testosterone levels were higher in the eggs of clutches produced with young males than in the eggs produced with older males, which supports the compensatory model. As this is a correlative finding, we have to be cautious in interpreting the results. One of the explanations may be that the correlation reflects a female's adaptive response to the likely lower quality of paternal care that young males might provide for their nestlings. Paternal care is important for reproductive success of collared flycatchers as indicated by reduced success of widowed or secondary females of polygynous males (Garamszegi et al., 2004a; Török et al., 1999). Young males often feed their nestlings less than do older males, possibly because they have lower foraging and feeding skills (Marchetti and Price, 1989), lack paternal experience (Forslund and Pärt, 1995), or are less willing to invest into current reproduction (Clutton-Brock, 1991). If maternal testosterone acts on collared flycatcher nestlings like in canaries (Schwabl, 1996), and black-headed gulls, *Larus ridibundus* (Eising et al., 2001), then begging intensity will be relatively higher in broods of young than older males. Because begging intensity stimulates parental care, and more so in males than in females (Ottoson et al., 1997), we propose that the deposition of more testosterone into eggs is a female compensatory tactic to elicit more feeding effort from young males. The comparison of the feeding activity of young and old males providing parental
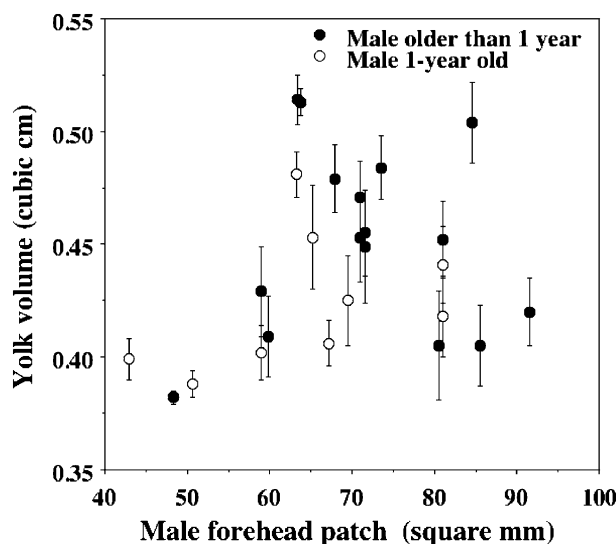

Figure 3
Yolk volume (clutch means with SEs) in relation to male attractiveness, the size of the male's white forehead patch, and male age.
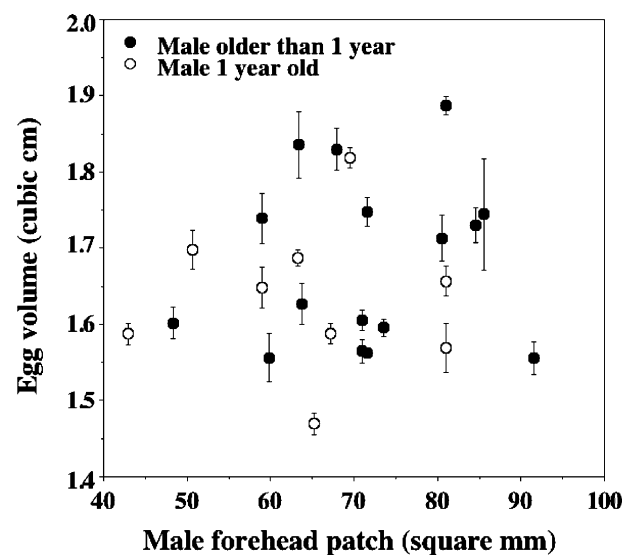

Figure 4
Egg volume (clutch means with SEs) in relation to male attractiveness, the size of the male's white forehead patch, and male age.

care to natural broods is not relevant for the verification of the presumption. The positive correlation between the mean testosterone level of the clutches and the feeding activity of the young males can only be demonstrated with data taken from cross-fostering experiments, when males face broods with different levels of begging activity.

An alternative explanation may be that female testosterone allocation depends on the onset of breeding (Mazuc et al., 2003), which at the same time influences the chances of females to have an old male as a partner. As the reproductive success of collared flycatcher females decreases with the advance of the breeding season (Garamszegi et al., 2004b; Wiggins et al., 1994), late-breeding females may increase their testosterone investment in order to reduce their reproductive handicap. In fact, our results supported this hypothesis, as we found a positive relationship between laying date and testosterone concentration in the eggs. In later broods, therefore, males may work harder in the feeding phase, thus compensating for the relative food shortage. As younger males arrive later and start to breed later, it may seem that females make their decision about testosterone investment with regard to male age, but in fact, the determining factor is the laying date. According to our results, the laying date is significantly correlated with the yolk testosterone level, which supports the importance of the timing of breeding. However, if we control for the laying date, male age still shows a strong association with the testosterone level of clutches. Therefore, both male age and laying date may be independent determinants of androgen allocation to eggs.

Other alternative explanations for the association of yolk testosterone with male age, such as phenotypic differences between females mated to young or older males (Groothuis and Schwabl, 2002; Pilz et al., 2003), are unlikely. First, yolk testosterone levels were not related to female morphological traits. Second, egg and yolk size were similar in females mated to young and older males, indicating that nutrient investment into eggs was similar. Third, female age was not correlated with yolk testosterone concentrations, rendering age-dependent female testosterone production and age-assortative mating unlikely causes for the apparent relationship with male age. Moreover, neither Pärt et al. (1992) in Sweden nor us in Hungary (regression analysis: $r^2 = .042$, $F = 0.598$, $p = .214$; $n = 38$ known-age pairs) were able to demonstrate age-assortative mating in the collared flycatcher. Thus, we are left with a female reproductive response that depends on male age and results in elevated testosterone levels in the eggs laid for young, inexperienced, and less attractive males. But why would not all females increase yolk testosterone to enhance male parental effort? Clearly there must be tradeoffs of high yolk testosterone for either the offspring or the female. Identification of the costs or tradeoffs is one of the current challenges in understanding the scope and limitations of maternal hormonal control.

Since maternal steroid hormones in the avian egg were first demonstrated (Schwabl, 1993) and discussed in relation to evolutionary processes such as parent-offspring conflict (Winkler, 1993), maternal hormonal control has been implicated also in mate choice and sexual selection (Gil et al., 1999), sex allocation (Petrie et al., 2001), population divergence, and local adaptation (Badyaev et al., 2002) and now sexual conflict. The relative importance of female hormonal control of these processes in different species likely depends on ecological conditions as well as on the benefits that females may gain. When considering these ultimate functions, we also have to keep in mind that steroid hormones are integral parts in the regulation of female reproductive physiology and behavior, and that these proximate regulatory functions will limit the scope of ultimate evolutionary functions.

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