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Maternal allocation of androgens and antagonistic effects of yolk androgens on sons and daughters

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Mothers can influence the phenotype of their offspring by adjusting the quality of their eggs in relation to sex and reproductive value of the progeny. Maternal androgens in the eggs of vertebrates may mediate such adaptive early maternal effects. However, the evolution of early maternal effects mediated by egg androgens may be constrained by the inability of mothers to differentially allocate androgens to eggs with a male or a female if androgens have different effects on sons and daughters. In this study, we increased the concentration of androgens in the eggs of barn swallows (*Hirundo rustica*) within the physiological range of variation and analyzed the effect on nestling growth and begging behavior. Egg androgens increased body size and mass of sons but reduced these characters in daughters when compared to two control groups in a repeated-measures analysis of variance of data collected at different ages. However, the differential effect of androgen on the two sexes was no longer significant when the analysis was restricted to the age of 12 days, when final body size is attained. In a second experiment, we tested whether mothers differentially allocated androgens to eggs with sons rather than daughters while manipulating a paternal secondary sexual character. Androgen concentration did not vary in relation to paternal ornamentation or embryo sex. Hence, antagonistic effects of egg androgens on sons and daughters may exist in the very early posthatching life and may constrain the evolution of adaptive maternal effects because mothers do not differentially allocate androgens in relation to embryo sex. *Key words:* begging display, early maternal effects, growth, *Hirundo rustica*, laying order, recruitment, viability. *[Behav Ecol 17:172–181 (2006)]*

Mothers have unique opportunities to influence the phenotype of their offspring by differentially allocating to their eggs substances that influence embryo development, having long-term effects on subsequent growth and performance (Mousseau and Fox, 1998). Such nongenetic maternal effects are expected to reflect adaptive strategies whereby mothers have evolved physiological mechanisms to adjust their investment in current progeny in relation to extrinsic factors including, for example, environmental conditions and paternal quality (Komdeur et al., 1997; Saino et al., 2002c). However, variation in egg quality may have differential effects on offspring of the two sexes, and this can impose a constraint on adaptive maternal effects, when mechanisms for differential allocation of egg components in relation to sex of the progeny have not evolved.

There is ample evidence among animal taxa that the quality of eggs can affect life-history traits of offspring, including their morphology, physiology, and viability (Mousseau and Fox, 1998). The eggs of oviparous vertebrates contain sex steroids of maternal origin (Adkins-Regan et al., 1995; Elf and Fivizzani, 2002; Gil et al., 1999; Schwabl, 1993, 1996a,b; Schwabl et al., 1997; Sockman and Schwabl, 2000). However, the effects of variation in the concentration of maternal androgens and estrogens in the egg on offspring development are still largely unknown. Studies where egg androgens were experimentally increased by in ovo injection have provided mixed evidence. Some studies have demonstrated positive effects of testosterone on embryonic muscular development, postnatal growth and survival, and social dominance rank (e.g., Eising et al., 2001; Lipar and Ketterson, 2000; Price and Ydenberg, 1995; Schwabl, 1993, 1996b). In contrast, other studies have shown that increased egg androgen concentrations delay hatching, reduce growth rate, and impair immunity, for example, by accelerating regression of a major primary immune organ, the bursa of Fabricius (e.g., Andersson et al., 2004; Henry and Burke, 1999; Sockman and Schwabl, 2000).

The apparently opposite effects of egg androgens on offspring traits related to fitness suggested by these studies raise the problem of optimal maternal allocation of hormones to eggs. This problem, however, may be complicated further if such hormonal mediators of maternal effects antagonistically affect offspring of the two sexes, whereby offspring of one sex benefit while those of the other sex suffer from high concentrations of yolk androgens. If mothers are incapable of differential allocation of androgens to the eggs in relation to embryo sex, this would result in concentrations of egg androgens deviating from an optimal level that would ensure superior performance for both sons and daughters. Only few studies have addressed the issue of the differential effects of egg androgens on offspring of the two sexes. Schwabl (1996b) found that exogenous testosterone promoted growth and begging behavior of captive canaries (*Serinus canaria*), with no differential effect on males and females. Henry and Burke (1999) showed reduced muscular development of female chickens as a result of injection of testosterone in the egg, while injection of an

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antiandrogen had the opposite effect. However, in the study by Henry and Burke (1999), postmanipulation hormone concentration appears to largely exceed the physiological levels, implying that the results of that study may not be relevant to the investigation of the effects of natural variation in egg androgen levels on progeny phenotype. Strasser and Schwabl (2004) demonstrated that injection of testosterone in the original egg enhanced the ability to dominate in dyadic encounters in both male and female captive house sparrows (*Passer domesticus*) and enhanced the expression of the black throat feather patch of males. Recently, Müller et al. (2005) have shown that injection of an antiandrogen in black-headed gull (*Larus ridibundus*) eggs enhanced body mass of male chicks whereas it reduced body mass and index of T cell–mediated immune response of females. In a study of the yellow-legged gull (*Larus michahellis*), injections of physiological amounts of testosterone in the egg yolk resulted in larger mortality of female than male offspring (Rubolini et al., in press).

Here, we first tested experimentally under field conditions whether yolk androgens had antagonistic effects on sons and daughters in a passerine bird, the barn swallow (*Hirundo rustica*), whose nestlings are not sexually dimorphic in body mass or size (Saino et al., 2002a). We injected immediately after laying testosterone and androstenedione, which are the most abundant androgens in barn swallow eggs, in the yolk thereby increasing the concentration of these hormones that occur naturally in eggs. These manipulations within the natural range of variation were used to test for a sex-related effect of exogenous androgens on somatic growth of the offspring and their food solicitation display (begging display).

Evolutionary theory of parental allocation predicts that parents should adjust their investment according to the reproductive value of offspring (Charnov, 1982; Fisher, 1930; Trivers and Willard, 1973). Mothers may thus be predicted to fine-tune androgen allocation in relation to the sex of individual offspring. Current theory of sexual selection posits that, by preferring males with exaggerated secondary sexual characters, females accrue indirect benefits for their sons in terms of sexual attractiveness or "good genes" for high viability for their progeny, if the expression of ornaments reliably reveals the genetic quality of males (reviewed in Andersson, 1994). Reproductive value of the offspring is therefore expected to increase with the expression of sexually selected characters of their father, and mothers should invest more in reproduction when their offspring are sired by males with large secondary sexual characters. Previous studies of birds have shown that transfer of maternal substances, including androgens (Gil et al., 1999), antigen-specific antibodies (Saino et al., 2002c), and carotenoids (Saino et al., 2002b) is influenced by the experimental manipulation of secondary sexual characters of the father. Recently, Gil et al. (in press) showed that barn swallow females mated to males with experimentally enlarged secondary sexual characters deposited more androgens in their first eggs compared to females mated to males with reduced ornaments. However, other studies did not find evidence for differential transfer of maternal hormones to the eggs in relation to paternal sexual attractiveness (e.g., Michl et al., 2005). Egg features such as size (Anderson et al., 1997; Cordero et al., 2000, 2001; Cunningham and Russell, 2000), concentration of androgens (Petrie et al., 2001; but see Eising et al., 2003; Elf and Fivizzani, 2002) or immune factors (Saino et al., 2003c), and growth patterns of oocytes (Young and Badyaev, 2004) have been shown to vary with sex of the embryo. Dominant leghorn hens transfer more testosterone to their eggs when they contain a son rather than a daughter (Müller et al., 2002; see also Eising et al., 2003). However, some studies have failed to find evidence of differential allocation of egg components (e.g., carotenoids; Saino et al., 2003c).

If maternally derived egg androgens enhance somatic growth of the offspring, as shown in some studies (Eising et al., 2001; Henry and Burke, 1999; Lipar and Ketterson, 2000; Schwabl, 1996b), eggs laid for males with naturally large or experimentally enlarged secondary sexual characters should contain larger concentrations of androgens as a result of an adaptive maternal favoritism for sons with high reproductive value. Differential maternal allocation may be expected because of the physiological costs experienced by the mother in terms, for example, of immune suppression, arising from elevated maternal androgen levels (Grossman, 1985; Schuurs and Verheul, 1990).

The barn swallows we studied here are sexually monomorphic except the length of the outermost tail feathers, which are longer in males than in females in our study population (Møller et al., 1995). Correlational and experimental studies have shown that females prefer males with relatively long tail feathers as social and extrapair copulation mates (Møller, 1994; Saino et al., 1997). In a second experiment, we therefore tested whether females allocated androgens to the egg yolk in different amounts in relation to the experimentally altered level of expression of tail ornaments of their mate and sex of the offspring. If mechanisms of differential allocation of androgens in relation to sexual ornamentation of the father and sex of the offspring have evolved, it can be predicted that the difference in androgen concentration between eggs with a male or a female embryo increases with the experimental level of sexual ornamentation of the father.

METHODS

The study was carried out in spring 2001–2002 at nine colonies near Milano, Northern Italy. Nests were visited every day allowing to mark the eggs according to laying order as a maximum of one egg is laid per day. In the egg-manipulation experiment, each egg in a clutch was randomly assigned to one of three experimental groups. We aimed at elevating the concentration of androgens in hormone-injected eggs to a final level within the physiological range of variation of the hormones. We therefore decided to inject an amount of testosterone and androstenedione that corresponded to 1 SD of the mean amount contained in an average yolk, weighing approximately 0.5 g, of a sample of 14 unincubated eggs (mean ± SD concentration [ng] per gram of yolk: testosterone: 2.73 ± 2.37; androstenedione = 8.24 ± 7.30) (Saino N., unpublished data). By doing so, we therefore increased the mean concentration of the hormones to a level 1 SD greater than the mean concentration observed in the sample of 14 eggs (see above), which were assumed to reflect the mean concentration existing in the two control groups of eggs (see below). The yolk of the first group of eggs was thus injected with 1.0 ng testosterone and 3.5 ng androstenedione in 4 μl sterile sesame oil. The amount of androstenedione (the only androgen assayed in the tail-manipulation experiment) we injected, however, was 1.4 times the SD of the mean amount contained in yolks of approximately 0.5 g from the two control groups in the second experiment (mean ± SD concentration [ng] per gram of yolk: 5.45 ± 5.14). Hence, the two sets of measures of androgens suggest that injection actually raised the postmanipulation concentration of androgens within the physiological range of variation in the vast majority of the eggs. The yolks of the second group were injected with 4 μl sterile sesame oil. The eggs of the third group were just handled. Eggs were randomly assigned to treatments using predetermined schemes where the three experimental groups were represented in a proportion of 2:2:1, and treatments were assigned randomly according to laying order (see also Saino et al., 2003a), assuming that the final clutch size corresponded to

the modal size in our study population (=five eggs). A scheme chosen randomly was then assigned to each particular clutch. In cases of clutches of six, the last egg was randomly assigned to one of the three groups.

Injection was done in the field from the egg acute pole, using 25-µl Hamilton syringes mounting 26-g needles above an intense light source to identify yolk position. The hole in the eggshell was sealed by glueing a small piece of swallow eggshell. We injected five additional eggs with a food dye and dissected them after they had been deep frozen after injection to check whether injection occurred in the yolk, and in all cases this was the case. Eggs were injected within the second day after laying. Because in this experiment assignment of eggs to treatments was randomized and the effect of androgen injection was assessed by comparing nestlings from injected eggs with their control siblings sharing the same nest environment and parents, we are confident that phenotypic variation among experimental groups of nestlings was largely unaffected by variation in ecological factors or parental phenotypic traits. Nests were frequently inspected around hatching to assign nestlings to their original egg. Nestlings that could not be assigned were excluded from analyses of morphological variables. Overall, 15 experimental clutches were discarded for different reasons, including total or partial failure, our own failure to assign most of the nestlings to their original treatment, early nestling mortality, or a combination of these conditions. The frequency of hatching failures putatively due to injection of the eggs could not be assessed precisely because some nestlings could not be assigned to their original egg and some eggs that possibly did not hatch disappeared from the nest before they could be identified. In addition, some dead hatchlings could have been ejected from the nest before we found them to have hatched. Based on the 37 broods that were actually included in the study (see below) and egg failures that we could record in the clutches that were discarded, we can estimate that hatching failures numbered 12% for control eggs ($n = 67$), 19% for sham-injected eggs ($n = 79$), and 21% for androgen-injected eggs ($n = 76$).

We measured body mass (g) on days 4, 7, and 12 posthatching (to the nearest 0.1 g), tarsus length (mm) on days 7 and 12 (to the nearest 0.05 mm), and left innermost tail feather length (mm) on day 12 (to the nearest 1 mm).

We analyzed data for 158 hatchlings from 37 broods, 151 of which could be assigned to their original treatment and sexed, including 75 females (21 from nonmanipulated, 22 from sham-injected, and 32 from androgen-injected eggs) and 76 males (24 from nonmanipulated, 29 from sham-injected, and 23 from androgen-injected eggs). Twelve (five females and seven males) out of the 151 nestlings, however, could not be assigned to their original egg but hatched from either of the two eggs belonging to the same experimental group. These nestlings could therefore be included in the analyses of the effect of treatment on nestling sex ratio and thus mortality but had to be excluded from the analyses of morphological variables because laying order of the egg from which they originated was unknown, and the value of this important covariate was therefore missing.

Manipulation of male secondary sexual characters

Males were randomly assigned to tail shortening by 20 mm, no manipulation, tail cutting and regluing without altering original length, or tail elongation by 20 mm (see Saino et al., 2002b for details). Tail manipulation occurred ten or more days before start of laying, after pair formation. We considered the first and fourth eggs from 12 clutches of tail-shortened fathers, 13 clutches of males that had their tail cut and reglued, 11 clutches of nonmanipulated males, and 13 clutches of tail-elongated males (see Saino et al., 2002b). We a priori decided to collect the fourth besides the first egg because a very large proportion of females lay at least four eggs in our study populations. The embryo of some eggs could not be sexed, and this accounts for differences between the number of eggs according to the sample of broods and the samples used in the analyses (see Results).

Androgen assay

Yolk concentration of androstenedione was determined by radioimmunoassay. Eggs were collected the second day after the laying of the last egg to ensure that the clutch was completed, and yolk was separated for androgen analyses. In the barn swallow, incubation starts the day of laying of the penultimate egg. Thus, when the eggs from the nests included in the tail-manipulation experiment were collected, the embryo was still at a very early stage of development. Yolks were weighed and homogenized in 1 ml distilled water by vortexing them with some glass beads. From a further 10× dilution of the sample, we took 100 µl for steroid extraction. Steroid extraction consisted in adding 3 ml of diethyl ether to the sample, vortexing for 1 min, and centrifuging for 5 min (4°C, 2000 rpm). The ether phase was decanted after snap freezing the tube in an alcohol bath at −30°C and evaporated under a stream of nitrogen. Extraction recoveries were calculated by adding tritiated steroids (1000 counts per minute [CPM]) (Amersham, Little Chalfont, UK) to the original samples. Because recoveries were all greater than 89%, we did not correct for individual extraction efficiency. We obtained specific steroid antibodies from a commercial supplier (P.A.R.I.S. laboratories, Compiegne, France). Cross-reactivity of A4 antiserum at 50% binding was 0.9% for dihydrotestosterone (DHT), 0.3% for T, and <0.1% for other steroids. Cross-reactivity of T antiserum at 50% binding was 12% for DHT and <1% for the rest of steroids tested. The intraassay coefficient of variation for androstenedione was 5.9%. Only one assay was performed, therefore preventing calculation of the interassay coefficient of variation. The lowest detectable concentration was 1 pg/mg.

Only androstenedione data were available for eggs included in the tail-manipulation experiment. However, testosterone concentration in nonincubated barn swallow eggs is strongly positively correlated with androstenedione concentration ($r = .80$, $n = 14$, $p < .001$) (Saino N, unpublished data).

In the analyses of the effect of tail manipulation on egg androgens, we used concentration rather than the absolute amount of the hormone as estimated by average yolk mass of eggs of different laying order. In fact, total mass of barn swallow eggs is known to increase with laying order (Ferrari et al., in press; Saino et al., 2004). However, variation in egg mass with laying order is due to variation of albumen content of the egg, whereas there is no significant relationship between yolk mass and laying order and, in particular, yolk mass of first eggs does not differ from yolk mass of fourth eggs of the same clutches (Ferrari et al., in press). Thus, androgen concentration should strictly reflect total amount contained in the yolk independently of egg-laying order.

Molecular sexing

To sex 4-day-old nestlings (egg-manipulation experiment) and embryos at very early stages of development (tail-manipulation experiment), we used the sex chromosome–linked avian CHD-1 gene (for details see Griffiths et al., 1998; Saino et al., 2002a). Nestling genetic material was obtained from small blood samples collected in capillary tubes on day 4. Embryos were accurately separated from other egg material under a microscope before DNA extraction and sexing.

Begging display

On day 10 after hatching, we recorded begging behavior of nestlings. Nestlings were placed together in a random position in a swallow nest and were stimulated to beg by intermittently, gently touching all parts of the nest rim with a Y-shaped stiff metal wire while video recording them for 1 min after the position of each nestling in the nest had been recorded (see Saino et al., 2000). Begging behavior was expressed as the number of times per minute (begging rate) that a nestling raised its head and opened its beak, as typical when nestlings solicit feeding from their parents (see Saino et al., 2000).

Statistical analyses

To test for an effect of treatment on sex ratio at age 4 days, reflecting a differential effect of egg treatment on viability of embryos and hatchlings of the two sexes, we first computed the total number of males and females for each egg treatment group within each brood. The proportion of males in each brood by treatment group was then subjected to a logistic regression analysis where brood and egg treatment were included as predictors. To correct for data overdispersion, the covariance matrix was multiplied by a heterogeneity factor (Pearson χ^2/df). Body mass data recorded at days 4, 7, and 12 posthatch and tarsus length data recorded at days 7 and 12 posthatch were analyzed in repeated-measures general linear mixed model (GLMM) analyses of variance (ANOVAs) with treatment of the original egg of each nestling and sex as fixed-effect factors, laying order of the egg as a covariate, and brood as a random-effect factor linking nestlings from the same nest. A factor nestling was also included to identify repeated measures from the same individual at different ages. Rectrix length recorded at age 12 days and begging behavior recorded at age 10 days were analyzed in single-measure GLMM with the same design as for the other characters. Phenotypic data of nestlings belonging to the same sex and experimental group within each brood were not averaged because laying order of the original egg of nestlings markedly influences offspring phenotype (see Results) and cannot be included in models where average phenotypic values of nestlings are analyzed. Two-way interactions between treatment, sex, and laying order were also initially included in the models. However, to derive minimum adequate models, we subsequently excluded the nonsignificant effects by a step-down procedure where at each step the term associated with the largest p was excluded (Crawley, 1993). Both initial and step-down models are reported in the Results. To represent mean values of body mass and size of the treatment × sex groups at different ages (that were significantly predicted by sex and treatment; see Results), we used least square means obtained from GLMM models where we included the phenotypic value at the focal age as the dependent variable, brood as a random effect, sex and treatment as fixed effects together with their interaction, and laying order as a covariate. Raw mean values were represented for variables that were not affected by sex and treatment. Egg-laying order and hatch order are strongly positively correlated ($r_s = .81$, $n = 139$, $p < .001$). To avoid inclusion of collinear covariates, only laying order was included in the models. Standard error of estimated parameters is given in parentheses. Analyses were run using SAS 8.2. To reduce the risk of incurring type I statistical errors, sequential Bonferroni correction for simultaneous tests on four phenotypic variables was applied by lowering the significance level to $0.05/4 = 0.015$. In analyses where we compared the two control groups or the sham-injection and androgen-injection groups, we lowered the significance level for two pairwise comparisons for each variable to $0.05/2 = 0.025$. Tests that were still significant after sequential Bonferroni correction are marked with an asterisk.

RESULTS

Consequences of in ovo androgen manipulation

We tested for an effect of egg treatment (no injection, sham injection, injection with androgens) on the proportion of males among 4-day-old nestlings by logistic regression analysis (see Statistical Analyses). Sex ratios showed nonsignificant variation among treatments ($\chi^2 = 5.19$, df = 2, $p = .075$). The sex ratio was slightly male biased in the group of nestlings from unmanipulated eggs (0.53, $n = 45$ nestlings) and sham-injected eggs (0.57, $n = 51$), while it showed an opposite deviation from parity among nestlings from androgen-injected eggs (0.42, $n = 55$). The proportion of male embryos in the barn swallow is 0.515 (see Saino et al., 2003c for the largest sample available), while the proportion of sons among nestlings is 0.492 (Saino et al., 2002a). These proportions do not differ from 0.5 ($p > .05$ in all cases). None of the within-group sex ratios deviated significantly from the null expectations based on sex ratios observed among embryos or nestlings or from parity (binomial $p > .10$ in all cases). Thus, there was no evidence of a statistically significant effect of androgen treatment on viability of eggs or nestlings differing between sons and daughters.

Repeated-measures ANOVAs showed that treatment per se had no significant effect on any nestling morphological character (Table 1). Sex had no significant effect on body mass and rectrix length, while its effect was marginally significant on tarsus length (Table 1). However, the effect of treatment on body mass and tarsus length depended on the sex of the nestlings, as shown by the significant interactions (Table 1). In addition, laying order strongly predicted all nestling phenotypic values (see also below). When the effect of the interaction between sex and treatment was removed from the models, treatment had no effect on nestling phenotype (details not shown).

The tests of within-subject effects in repeated-measures analyses neither showed significant interaction between age and sex or treatment on body mass or tarsus length nor significant three-way interactions between age, sex, and treatment (Table 1). These results imply that the differential effect of treatment on nestlings of the two sexes did not vary at different ages. Therefore, according to repeated-measures ANOVA models presented in Table 1, the differences in the effects of treatment between males and females were consistent at different ages.

A step-down procedure of exclusion of nonsignificant terms from repeated-measures analyses (see Statistical Analyses) led to models where sex had a marginally nonsignificant effect on body mass ($F = 4.36$, df = 1,110, $p = .04$) and a nonsignificant effect on tarsus length ($F = 1.50$, df = 1,113, $p = .22$) (Figure 1). In these models, the interaction between sex and treatment had a highly significant effect on body mass ($F = 10.64$, df = 2,106, $p < .001^*$) and tarsus length ($F = 5.39$, df = 2,108, $p = .006^*$) (Figure 1). Laying order negatively predicted body mass ($F = 97.63$, df = 1,101, $p < .001^*$, coefficient = −0.58 (0.06)) and tarsus length ($F = 23.01$, df = 1,102, $p < .001^*$, coefficient = −0.10 (0.02)). The step-down model for rectrix length included only laying order with a highly significant negative effect ($F = 57.83$, df = 1,106, $p < .001^*$, coefficient = −1.10 (0.14)). Thus, there was no effect of sex or treatment on tail feather growth (Figure 2). The effect of the interactions between laying order and sex or egg treatment was nonsignificant in all analyses and was therefore excluded from step-down models.

Table 1

Mixed-model repeated-measures (for body mass and tarsus length) or single-measure (for rectrix length and begging rate) ANOVAs in relation to sex and egg treatment (fixed effects) and laying order of the original egg (covariate)

	Numerator df	Denominator df	F	p
Body mass				
Between-subjects effects				
Sex	1	101	2.66	.11
Egg treatment	2	103	0.86	.43
Laying order	1	97.3	95.48	<.001*
Sex × egg treatment	2	103	10.62	<.001*
Sex × laying order	1	103	0.56	.46
Treatment × laying order	2	104	1.09	.34
Within-subjects effects				
Age	2	264	727.55	<.001*
Age × sex	2	264	0.68	.54
Age × treatment	4	264	0.08	.98
Age × laying order	2	264	3.86	.02
Age × sex × treatment	4	264	1.28	.28
Tarsus length				
Between-subjects effects				
Sex	1	104	5.35	.022
Egg treatment	2	106	0.64	.53
Laying order	1	99.6	25.20	<.001*
Sex × egg treatment	2	105	5.61	.005*
Sex × laying order	1	106	3.80	.054
Treatment × laying order	2	107	0.66	.52
Within-subjects effects				
Age	1	132	21.37	<.001*
Age × sex	1	132	1.19	.28
Age × treatment	2	132	0.27	.77
Age × laying order	1	132	6.35	.01
Age × sex × treatment	2	132	0.23	.80
Rectrix length				
Sex	1	100	2.61	.11
Egg treatment	2	102	0.36	.70
Laying order	1	98	53.35	<.001*
Sex × egg treatment	2	101	1.33	.27
Sex × laying order	1	101	2.64	.11
Treatment × laying order	2	102	1.66	.19
Begging rate				
Sex	1	115	0.47	.49
Egg treatment	2	117	0.29	.75
Laying order	1	106	0.32	.57
Sex × egg treatment	2	117	0.15	.86
Sex × laying order	1	117	0.18	.67
Treatment × laying order	2	118	0.06	.94

In these models, "brood" was entered as a random-effect factor linking nestlings from the same brood. Asterisks indicate tests that are significant after sequential Bonferroni correction.

To test whether the observed differential effect of egg treatment in relation to sex on body mass and tarsus length was due to egg injection per se or androgen injection, we ran separate analyses in which we compared the two control groups or the sham-injection and the androgen-injection groups. The same step-down repeated-measures ANOVAs we presented above as applied to all groups, when applied to the two control groups showed that the interaction between sex and egg treatment was nonsignificant for both variables (body mass: $F = 0.56$, df = 1,61.2, $p = .46$; tarsus length: $F = 0.00$, df = 1,63.6, $p = .95$). In these models, the effect of treatment remained nonsignificant ($p > .05$ for both variables), while the effect of sex was highly significant on both body mass ($F = 16.24$, df = 1,64, $p < .001^*$) and tarsus length ($F = 9.74$, df = 1,66.8, $p = .002^*$). These results show that among nestlings from control eggs females were larger than males (Figure 1).

In the analysis of nestlings from sham-injected and androgen-treated eggs, a significant interaction between the effects of sex and treatment was found for body mass ($F = 8.08$, df = 1,72.2, $p = .006^*$) and tarsus length ($F = 7.54$, df = 1,71.6, $p = .008^*$), whereas the main effects of treatment or sex were not significant ($p > .05$). Thus, the significant effects of the interaction between egg treatment and sex observed in the analyses including all experimental groups were mainly due to a differential effect of androgen injection on nestlings of the two sexes rather than to a differential effect of egg injection per se. Androgen injection appeared to enhance body mass and tarsus length of male offspring, whereas it had an opposite effect on females (Figure 1).

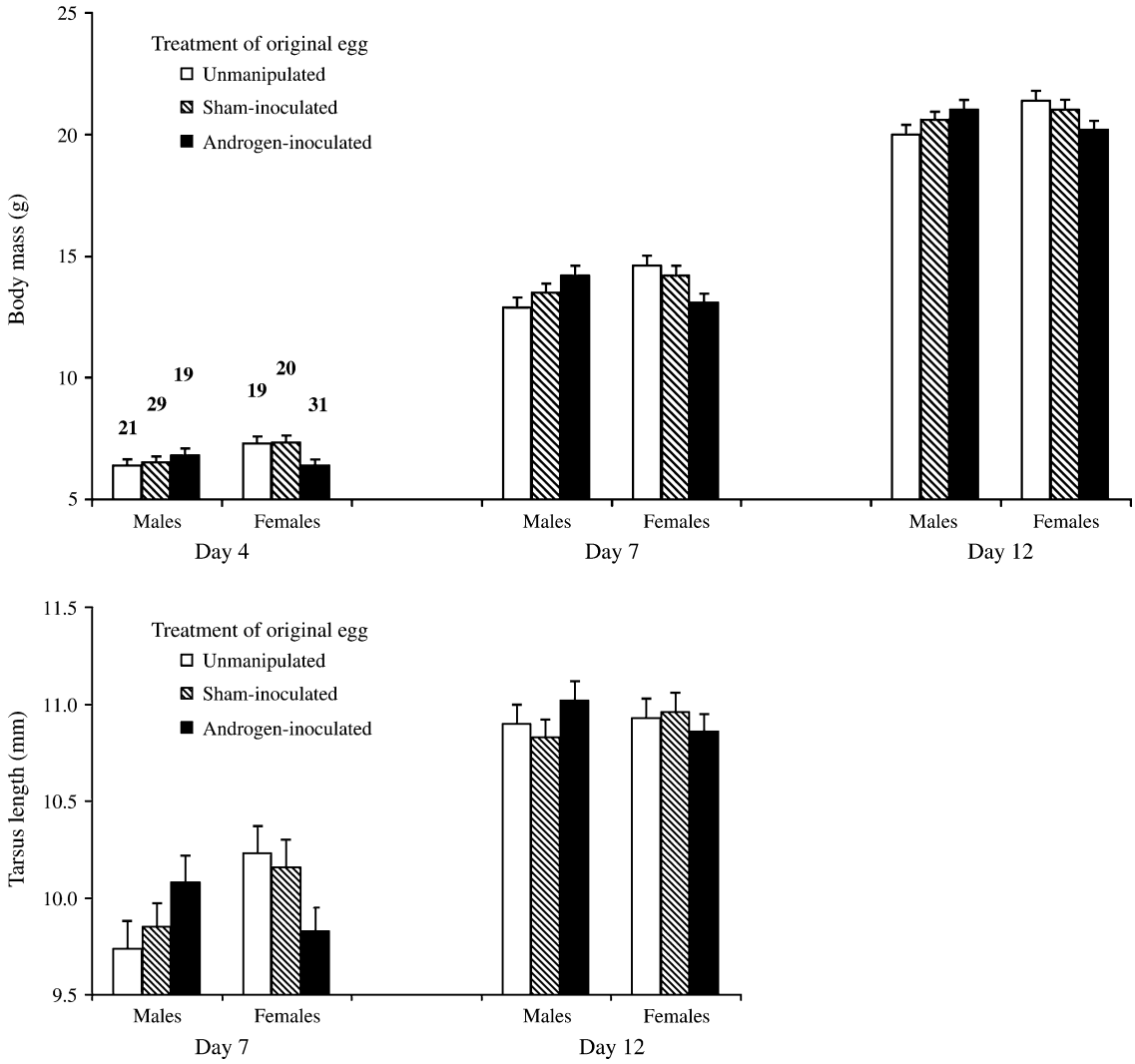

Figure 1
Least square means (+SE) of body mass (g) and tarsus length (mm) at different ages obtained from ANOVAs with brood as a random-effect factor, egg-laying order as a covariate, and sex of offspring, treatment (fixed effects), and their interaction (see Statistical Analyses). The number of nestlings in each sex by treatment group is shown.

Finally, we used step-down single-measure ANOVAs to test whether androgen treatment had a differential effect on body size and mass of male and female nestlings at day 12, when tarsus is fully grown and maximum body mass is attained. Neither body mass nor tarsus length was found to be affected by the interaction between sex and treatment in the analysis of nestlings from the two control groups (step-down models, details not reported). The analysis restricted to nestlings from the sham-injection and androgen-injection groups showed marginally nonsignificant effects of the interaction between sex and treatment on body mass ($F = 4.05$, df = 1,74.7, $p = .048$) and tarsus length ($F = 2.90$, df = 1,77.3, $p = .093$).

Thus, while repeated-measures ANOVAs showed that the significant differential effect of treatment on male and female offspring did not vary at different ages, the analysis at day 12 provided marginally nonsignificant evidence for a sex-related effect of treatment on body mass.

Begging rate was not significantly affected by egg treatment, sex of offspring, laying order, or the two-way interactions (Table 1; Figure 2).

Egg androgens in relation to paternal ornamentation

In the tail-manipulation experiment, we analyzed androstenedione concentration (which is strongly positively correlated with testosterone concentration; see Methods) in relation to offspring sex and male tail length. A nested ANOVA with sex and laying order as factors, where the effect of mother was hierarchically included in the effect of tail treatment, showed no significant variation of androstenedione concentration in relation to tail treatment ($F = 1.17$, df = 3,45, $p = .33$), sex of offspring ($F = 0.30$, df = 1,31, $p = .59$), and the interactions between offspring sex and paternal tail manipulation ($F = 0.63$, df = 3,31, $p = .60$) or laying order ($F = 2.14$, df = 1,31, $p = .15$; Figure 3). These results show that mothers did not differentially allocate androstenedione in relation to the independent or combined (interaction) effects of male sexual attractiveness and sex of the offspring. The exclusion of sex from the model, which allowed the inclusion of 15 additional eggs whose embryo (if any) could not be successfully sexed, confirmed the lack of effect of paternal tail manipulation on androstenedione concentration ($F = 0.39$, df = 3,48, $p = .76$)

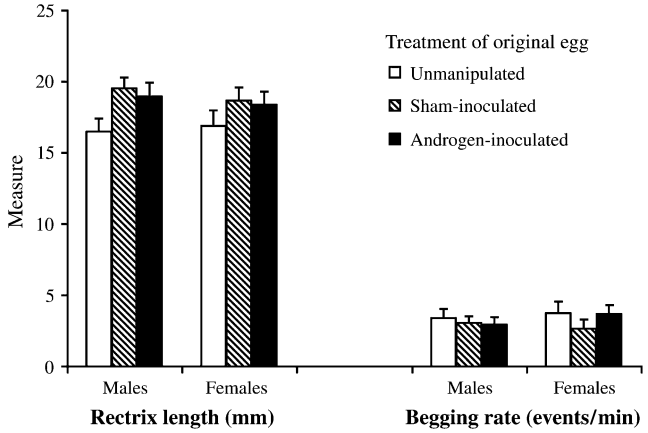

Figure 2
Raw mean (+SE) values of rectrix length and begging rate of nestlings in relation to sex and treatment of the original egg. See Figure 1 for sample sizes of each sex × treatment group.

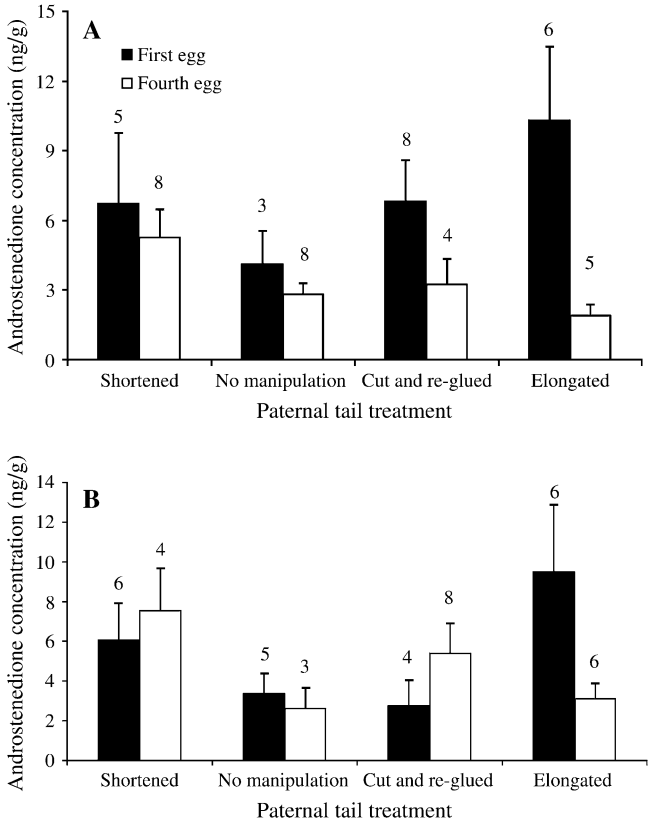

Figure 3
Mean (+SE) androstenedione concentration in eggs with a male (A) or a female (B) embryo in relation to laying order and manipulation of tail length of the male attending the nest. Numbers are number of eggs.

and showed that the concentration of the hormone was larger among first than fourth eggs ($F = 10.74$, df = 1,48, $p = .002$; least square means (ng/g): first eggs, 7.45; fourth eggs, 4.49). The effect of the interaction between laying order and tail treatment was not statistically significant ($F = 1.89$, df = 3,48, $p = .14$).

DISCUSSION

In the first experiment, we tested for antagonistic effects of yolk androgens on sons and daughters by experimentally manipulating androgen concentration in eggs within the natural range of variation of these hormones. Repeated-measures ANOVAs showed a differential effect of androgens on phenotypic values of nestlings of the two sexes. Sons from eggs injected with androgens were larger, while daughters were smaller than their siblings from two control groups. However, the analyses restricted to age 12 days, when tarsus is fully grown, did not show significant effects of egg hormone treatment. The effect of hormone injection was tested by comparing nestlings from injected eggs with their control siblings sharing the same parents and nest environment. Therefore, we are confident that we could effectively control for environmental effects that may otherwise have confounded the results. It should be emphasized, however, that in the present as well as in other studies where androgens have been injected in the yolk experimental treatment may not exactly mimic the effect of elevated levels of maternal androgens. The reason is that injection in a small region of the yolk does not reproduce the natural pattern of variation of androgen levels among different yolk layers (Lipar et al., 1999). However, even if that is the case, this experimental shortcoming cannot account for the significant treatment by sex interactions.

Androgen treatment did not significantly affect the sex ratio of young nestlings. The result of the logistic regression analysis on the effect of treatment on sex ratio, however, was marginally nonsignificant, suggesting that androgens may have had a weak, differential effect on mortality of sons and daughters. It should be noted that the relatively large, nonsignificant effect of treatment on sex ratio was partly due to the female-biased sex ratio among nestlings from eggs injected with the control solution, which, importantly, did not significantly deviate from parity or sex ratios observed among embryos or nestlings in previous studies in our study population (Saino et al., 2002a, 2003c). Because it seems unlikely that injection of oil caused larger mortality of sons than daughters, thus causing a female bias in the sex ratio of nestlings from sham-injected eggs, we are convinced that there was no effect of egg treatment on nestling sex ratio as mediated by differential mortality of the two sexes at the embryonic or early nestling stages.

Local recruitment of the offspring into the breeding population in our study area is small, owing to high natal dispersal, particularly among females. Therefore, we could not directly investigate the effect of manipulation of androgen concentration in the egg on viability as reflected by local recruitment. In addition, androgen egg treatment could also affect postfledging behavior, implying that differential local recruitment in relation to treatment of the original egg, if documented, could reflect an effect on dispersal behavior rather than survival. In our study population, body size as reflected by tarsus length predicts probability of local recruitment of both sexes (Saino N, in preparation). This result is similar to what has been found in other birds (e.g., Alatalo and Lundberg, 1986). Local recruitment has been often assumed to reflect offspring viability (e.g., Kruuk et al., 2002; Pettifor et al., 2001; Schmoll et al., 2003). Under the untested assumption that this applies also to the barn swallows we studied, the present results may suggest that physiological doses of exogenous androgens antagonistically affect the development of a phenotypic character of sons and daughters related to viability. However, it should be emphasized that a significant differential effect of egg treatment on growth of males and females was short lived and emerged only when morphological data from the whole nestling period were considered,

whereas this effect was not statistically significant when the analysis was restricted to data recorded at age 12 days.

Diverse mechanisms may have mediated the differential effect of egg androgens on sons and daughters (see Badyaev, 2002). Egg androgens may directly affect offspring growth. In vertebrates, somatic growth and thus body size are largely modulated by growth hormone (GH), which is secreted by the anterior pituitary in a sex- and age-dependent manner. Release of GH depends on two hypothalamic hormones that are synthesized and secreted under control of gonadal steroids. Exposure to steroids already during early ontogeny can influence GH-dependent growth in the two sexes by diverse mechanisms (Badyaev, 2002). Gonadal hormones can modulate the expression of pituitary receptors for hypothalamic stimulators of GH release and thus produce sexual differences in GH profiles (Kamegai et al., 1999). Prenatal exposure to steroids can imprint the sex-specific sensitivity of the pituitary to the hormones controlling the release of GH (Gatford et al., 1998). Sex steroids can modulate hypothalamic neuropeptides (i.e., somatostatin and GH-releasing hormone) that regulate GH release from the pituitary, and early exposure to sex steroids can influence the generation of GH secretory patterns (Chowen et al., 1996; Veldhuis and Iranmanesh, 1996). In addition, sexual differences in steroid milieu in early development can affect the expression of hormone receptors and hormone-secreting cells with persisting long-term consequences for sensitivity to hormones in the two sexes (Brandstetter et al., 2000; Lopez et al., 1995). Increased exposure to prenatal androgens determined by in ovo androgen injection may have interfered with any of these mechanisms of growth control in a different way in the two sexes, resulting in antagonistic effects on somatic growth.

A partly different interpretation of the effect of androgens is that they influenced the expression of nestling behavioral traits relevant to parent-offspring communication (Schwabl, 1996b) or aggressive behavior (Schwabl, 1993) and thus sib-sib scramble competition for food. The effect of androgens on the expression of "begging" displays or the ability to outperform siblings may differ between sons and daughters and ultimately result in a positive effect of egg androgens on growth of sons compared to daughters. However, we found no evidence for an effect of egg treatment on begging behavior. This suggests that either androgen injection had an effect on another component of "begging display" we did not measure (e.g., quality of begging calls) or that a differential effect of egg androgens on growth of offspring of the two sexes mediated by behavioral characters has arisen via an effect on sib-sib scramble competition for food. Direct effects of androgens on nestling growth and indirect effects mediated by behavior may obviously interact because an initial advantage for sons from androgen-injected eggs may subsequently be amplified by the greater ability of large nestlings to compete for food.

The result that control daughters were larger than control sons was unexpected because we have previously shown that in a sample of unmanipulated broods there is no sexual dimorphism in either body mass or size (Saino et al., 2002a). Control daughters may have received more care than control sons because they may have been recognized by parents as being of relatively high quality compared to their sisters hatched from androgen-injected eggs. This would require that parents can discriminate among offspring in relation to their sex. In fact, male and female nestling barn swallows have been shown to differ in sonagraphic features of begging vocalizations, at least late in the nestling period (Saino et al., 2003b), and parents may thus also have an acoustic clue to discriminate between their offspring in relation to sex already at early stages of the nestling period.

Antagonistic effects of egg components on sons compared to daughters may thus constrain the evolution of adaptive maternal effects, constituting the selection pressure that ultimately may result in the evolution of mechanisms of allocation that depend on the sex of offspring. Previous studies of birds suggest that females can fine-tune egg quality, in terms of androgen concentration, according to the sex of the embryo (Petrie et al., 2001; but see Eising et al., 2003). Here, we tested for such differential allocation of androgens to eggs in relation to sex of the embryo, while experimentally manipulating the size of a male secondary sexual character. We predicted that females should transfer more androgens to their eggs when these contained a son sired by a male with experimentally enlarged secondary sexual characters. Contrary to this expectation, we found no effect of paternal ornamentation and offspring sex on androgen concentration. However, it must also be emphasized that the sample size for some of the embryo sex by laying order by paternal treatment groups was small, and this could have inflated the risk of incurring type II statistical errors. These results must therefore be taken cautiously.

Gil et al. (in press) have found that in a Spanish barn swallow population the concentration of androstenedione deposited by females in their first eggs increases when the tail ornaments of their mate are experimentally enlarged. Thus, in the population studied by Gil et al., females adjusted egg quality in relation to sexual attractiveness of their mate in an apparently adaptive way as high androgen concentration may boost son development (Gil et al., in press). The difference in the effect of tail length manipulation on androstenedione concentration between the present and Gil et al. (in press) studies could be due to different power of the tests as the Spanish study was based on a larger sample of first eggs and the sign of difference in androstenedione concentration between the eggs laid by females mated to tail-elongated and tail-shortened males was consistent in the two studies. However, by injecting physiological doses of androgens in the eggs, we showed that females might not accrue a net benefit in terms of offspring phenotypic quality because of the antagonistic effects of androgens on sons and daughters. Lack of response to tail manipulation of the male mate in the Italian barn swallow population may thus suggest that mothers are optimally transferring androgens to the eggs, under the constraint imposed by sex-related androgen effects on the offspring and maternal inability to differentially provision the eggs carrying a male or a female with androgens. Along this line of reasoning, the effect of tail manipulation on hormonal egg quality observed in the Spanish population leads us to predict that an experimental increase of androgen concentration in the eggs in that population could result in a net benefit for females in terms of offspring phenotypic quality.

Physiological mechanisms of differential allocation of androgens to eggs dependent on the sex of the offspring do not appear to have evolved in the barn swallow. Given that androgens have antagonistic effects on sons and daughters at least during the early life stages, selection for such differential allocation of maternal androgens may occur. However, this inference relies on the untested assumptions that the antagonistic effects of androgens on males and females while in the nest have nonnegligible effects on individual fitness and that the sex-specific effects of androgens (either positive or negative) on body size are not compensated by contrasting effects on other traits (e.g., Navara et al., 2003). Furthermore, selection for differential allocation of maternal androgens may be constrained by physiological costs of modulation of hormone egg content to the mother.

We also considered whether extrapair fertilizations could have affected the results of our analyses. In the barn swallow,

approximately one-third of the nestlings are sired by a male different from the social mate of the mother (Saino et al., 1997). Thus, some fraction of the embryos we considered was probably sired by an extrapair male. In the present study, parentage of the offspring was not assessed. A previous study of the barn swallow showed no differential allocation to sex in relation to paternity or expression of secondary sexual characters of the parental or biological father (Saino et al., 1999). Thus, the present results suggest that the occurrence of extrapair fertilizations should not have biased the analyses, as does previous evidence for the lack of differential allocation to offspring of either sex in relation to paternal ornamentation.

Antagonistic effects of yolk androgens on sons and daughters may have important general implications. First, sex-specific effects of egg androgens on particular characters will cause the phenotype of either sons or daughters or both to deviate from the optimum because fitness benefits to parents mediated by the beneficial effects of relatively high levels of androgens to offspring of one sex will have to be traded against the detrimental effects on offspring of the other sex (Chippindale et al., 2001). Second, biased sex-allocation strategies by individual mothers will be favored by natural selection (West and Sheldon, 2002) because mothers producing offspring of one sex will have more opportunities of adaptively adjusting egg quality in relation to the sex of their offspring compared to mothers producing offspring of both sexes in the same brood. In fact, we previously documented a significant excess of unisex broods in the barn swallow compared to the binomial null expectation, suggesting that differential sex-allocation strategies have evolved in our population of barn swallows (Saino et al., 2002a). Third, any extrinsic (e.g., social) or intrinsic (e.g., general condition or age) factors affecting maternal androgen levels and thus transfer of hormones to eggs will influence the relative fitness benefits of producing offspring of either sex, resulting in covariation between progeny sex ratio and ecological conditions or maternal phenotype (Saino et al., 2002a; West and Sheldon, 2002).

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