

Sex differences in yolk hormones depend on maternal social status in Leghorn chickens (*Gallus gallus domesticus*)

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Maternal hormones are known to be present in avian eggs and can have beneficial effects on chick development. Recently, differences in avian yolk steroid concentrations between the sexes have been demonstrated, and in this context steroids have been proposed to be part of the avian sex-determining mechanism. In our study, we show that it is very unlikely that androgen concentrations alone are the decisive part of the sex-determining mechanism. We found that sex-specific differences in the yolk hormones strongly depend on the social rank of the mother. First, dominant females, but not subordinate females, allocated significantly more testosterone to male eggs than to female eggs. Second, subordinate females increased the testosterone concentrations of female eggs.

This pattern of yolk hormone deposition can be functionally explained. In polygynous species such as the chicken, reproductive success is more variable in males than in females. Parental investment in sons or daughters is therefore expected to occur in direct relation to parental rearing capacities. We found that the social status of a hen was indeed negatively correlated with her maternal capacities (for example, body mass, egg mass). Differential androgen deposition might thus provide a mechanism for adaptive maternal investment depending on both the sex of the egg and the social status of the mother.

Keywords: maternal effects; yolk androgens; social dominance; sex ratio; domestic chicken

1. INTRODUCTION

Avian eggs contain hormones of maternal origin that seem to reflect the hormonal state of the female during egg production (Schwabl 1996a, 1997) and have been shown to influence the development and phenotype of the offspring (Adkins-Regan *et al.* 1995; Schwabl 1993, 1996b; Lipar & Ketterson 2000). In particular, maternal androgens are known to increase competitiveness in the nestling, as well as in the juvenile stage, through higher aggressiveness and enhanced growth rate (Schwabl 1993, 1996b; Eising *et al.* 2001).

Systematic variation of maternal hormones, both within clutches (e.g. Schwabl *et al.* 1997; Gil *et al.* 1999; Lipar *et al.* 1999; Eising *et al.* 2001; French *et al.* 2001; Royle *et al.* 2001) and between clutches (e.g. Schwabl 1996a, 1997; Gil *et al.* 1999; Groothuis & Schwabl 2002; Wittingham & Schwabl 2002) has been reported. The former has mainly been discussed as a possibility for the mother to compensate for detrimental effects of hatching asynchrony (Schwabl 1996a; Lipar & Ketterson 2000; Eising *et al.* 2001; but see Sockman & Schwabl 2000). The second level of variation suggested that social factors have a strong impact on the androgen levels of a clutch (social environment: Schwabl (1996a, 1997); Reed & Vleck (2001); Groothuis & Schwabl (2002); Wittingham & Schwabl (2002); male attractiveness: Gil *et al.* (1999)).

In addition, Petrie *et al.* (2001) recently showed that for the peafowl (*Pavo cristatus*), yolk steroids were also

allocated differentially in relation to the sex of the embryo. Since the yolk is provided with hormones before the sex is determined (Sturkie 1986, pp. 403–420), Petrie *et al.* (2001) suggested that the steroids in the yolk itself may influence sex-chromosome segregation at the first meiotic division. Thus, sex-specific differences in yolk hormone concentrations might be a consequence of these processes.

However, we consider four possible confounding factors in Petrie *et al.*'s study. First, hormone levels in the peafowl were determined after 10 days of incubation and might therefore not represent maternal hormone allocation. This is supported by the results of Schwabl (1993) who found no sex-specific androgen deposition in freshly laid eggs of the canary. In size-dimorphic species (for example, the peafowl and other *Galliformes* such as our study species) in particular, male and female embryos may consume maternal steroids at a different rate. Second, endogenous production of the sex steroids of male and female embryos differs (Woods *et al.* 1975) and subsequently may be passed through into the yolk (Jennings *et al.* 2000). With an increase in incubation time, both might lead to a (secondary) sex difference in yolk hormones. Third, housing four peahens with a male in a cage, as in the study of Petrie *et al.* (2001), most probably leads to the establishment of a social hierarchy (e.g. Banks 1956; Guhl 1962). As it has been shown that the female endocrine state varies with her social position (Allee *et al.* 1939; Frank *et al.* 1985; Batty *et al.* 1986), we expect that this effect might be found in the eggs, via the yolk hormones. The yolk hormone contents of different hens within one group might therefore vary systematically. Because the number and weight of eggs are not evenly distributed over the different ranks (Leonard & Weatherhead 1996), a random

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selection of single male/female eggs taken from one cage, without taking the female into account, might lead to a biased sample.

In addition, social factors might cause sex-specific within-clutch variation in yolk androgens. In polygynous mating systems such as in *Galliformes*, male mating success is more variable than female mating success (e.g. Guhl & Warren 1946; Graves *et al.* 1985; see also Clutton-Brock *et al.* 1984). Hence, optimal parental investment should differ with sex, depending on parental rearing capacities (Trivers & Willard 1973). As social status has been shown to positively affect maternal rearing capacities (Collias *et al.* 1994; Leonard & Weatherhead 1996), dominant females should invest more in male than female offspring, whereas subordinate females should invest more in daughters. This might result in a shifted sex ratio or lead to a differential resource allocation of, for example, yolk androgens, which provide a mechanism to influence offspring phenotype.

To evaluate these potential confounding factors that might have influenced Petrie *et al.*'s study, we conducted an experiment in which these factors were taken into account. We measured yolk androgen concentrations in the eggs of White Leghorn chickens (*Gallus gallus domesticus*) in relation to the sex of the embryo after only three days of incubation. Thereby we minimized the possibility of secondary sex-specific processes affecting yolk hormone concentrations. This allows us to interpret any sex-specific differences in yolk hormones as definitely reflecting maternal allocation. We also investigated the relationship between the social rank of the mother and yolk androgen deposition.

2. MATERIAL AND METHODS

(a) *Animals*

Twenty-five individually colour-ringed White Leghorn chickens were housed in five groups, which comprised four females and one male, for approximately two months before the experiment began. Thus, a stable social hierarchy was established at the beginning of our experiment. Each pen measured 5 × 10 m with outside and inside areas (natural daylight 12 L : 12 D (March)). The outside areas of the pens were only separated by a chicken-wire partition. Thus, acoustic and optical, but no direct contact between groups was possible. Food and water were provided *ad libitum*. Every pen contained a single nest-box where all hens laid their eggs. Only a single female could enter this box at one time to lay her egg. At the start of the experiment, all females were weighed.

(b) *Behavioural observations*

The dominance hierarchy within each group was investigated by daily 20 min observations for a period of three weeks. All observations were conducted at the same time of day (13.00–15.00 h), mostly combined with the feeding of a favourite food (e.g. mealworms). The order in which the cages were observed was randomized each day. During the feeding, all agonistic interactions between the females were recorded. The dominance hierarchy was based on the proportion of winning or losing interactions with other females. A female lost an interaction if she fled when another hen pecked, chased or threatened her. Subsequently, the birds were ranked (highest rank: 1, dominant; lowest rank: 4, subordinate) according to which individuals they

dominated. A hen was considered to be dominant to another if it won more interactions than it lost with that hen. In addition, the percentage of feedings in which a hen obtained a share of the chosen food was scored.

(c) *Egg collection*

During the light period of the three weeks of the experiment, the identities of the females who entered the nest-box were recorded. After a hen had left, we immediately checked whether an egg had been laid. The egg was then removed and individually marked with a non-toxic marker. At the end of the day, all eggs were measured and weighed and subsequently placed in an incubator at 37.5 °C with 60% humidity. After incubation for 72 h, all eggs were weighed again and stored at –20 °C. In total, 182 eggs were collected. Laying order cannot be a confounding factor in this study since the eggs of each hen were collected randomly with respect to day within the three-week period. Laying order is also unlikely to be important in our birds since the hens lay eggs almost every day of the year, and not in separate clutches.

(d) *Molecular analyses*

For molecular analysis, the collected eggs were defrosted and the yolk and embryo were subsequently separated. Both were separately prepared for (i) molecular sexing (embryo); and (ii) androgen assays (yolk). The embryos were placed in Eppendorf tubes containing 100% ethanol and refrozen at –20 °C. The yolks were homogenized with 1 ml of water per gram of yolk and stored again at –20 °C until the analysis.

Because we were interested in sex differences, only fertilized eggs were used for further measurements ($n = 120$). Unfortunately, in one of the groups the cock was infertile, therefore this cage was excluded from the analysis of yolk hormones and sex ratios. Data from all five cages were used for all other analyses.

(e) *Molecular sexing*

Approximately 1 µg of tissue from each embryo was used for Chelex resin-based DNA extraction (Walsh *et al.* 1991). Subsequently, 2 µl of the DNA solution obtained were used for the polymerase chain reaction (PCR) to amplify a part of the *chd-w* gene in females and the *chd-z* gene in both sexes (for details, see Griffiths *et al.* 1998). The amplified products were separated in 2.5% agarose gels containing 0.005% ethidiumbromide and subsequently visualized under UV light. Based on the presence of the PCR products, embryos were designated as male (*chd-z* gene product only) or female (*chd-z* gene as well as *chd-w* gene products). This method has been developed and validated for domestic chickens (Griffiths *et al.* 1998) and we confirmed our results for all of our adult birds, obtaining a 100% correct outcome.

(f) *Androgen assays*

When available, at least three male eggs and three female eggs per hen were selected for hormone analysis. These eggs were randomly chosen throughout the laying period. The selected samples were defrosted and *ca.* 150 mg of the yolk/water emulsion were used for the subsequent analysis. All extractions and radioimmunoassays (RAI) were carried out following a slightly modified standard procedure according to Schwabl (1993). Briefly, samples were extracted twice with 4 ml of petroleum ether/diethylether (30/70%), followed by precipitation with 90% ethanol to remove neutral lipids. Subsequently, the hormones were separated on diatomaceous earth chromatography col-

Table 1. Social dominance hierarchy for the five cages based on direct interactions between the hens (number of won fights/number of fights) and participation during the feeding (frequency, percentage of feedings in which a hen got at least one food item).

cage	rank	1	2	3	4	frequency	female weight (g)
6	1		27/31	6/6	6/6	100	1915
	2			7/7	6/6	75	1815
	3				1/1	25	1886
	4					13	1698
7	1		10/13	11/17	8/11	100	2084
	2			21/27	14/23	100	1639
	3				26/36	100	1642
	4					0	1512
8	1		6/6	2/3	3/3	100	1775
	2			9/9	32/48	92	1706
	3				11/16	25	1560
	4					8	1510
9	1		35/35	66/67	24/30	100	1570
	2			49/55	12/13	75	1543
	3				6/6	75	1626
	4					0	1220
10	1		18/21	21/33	14/18	80	1968
	2			12/21	4/5	60	1956
	3				2/2	0	1988
	4					0	1513

umns. Androstenedione and testosterone concentrations were measured in double-competitive binding RIAs with tritiated hormone (NEN, The Netherlands) and hormone-specific antibodies (Endocrine Science, USA). The average recovery was 66% for androstenedione and 50% for testosterone. The inter-assay coefficients of variation were 17% for androstenedione and 11% for testosterone; intra-assay variation was 15% for androstenedione and 14% for testosterone.

(g) Statistical analyses

Testosterone, but not androstenedione, concentrations were normally distributed. For androstenedione, therefore, log-transformed values were used in our analysis. Yolk androgen levels, egg weight, yolk weight and embryo weight were analysed using hierarchical linear modelling in the MLWIN program v. 1.1 (Rasbash *et al.* 2000). This method allows analyses of variances and covariances considering the nested relationship of different chickens in a cage, and repeated measures of the same hen. Significance was based on a two-tailed *t*-test. The following variables were tested in a backward elimination procedure: social rank, sex (of the embryo), female body weight, egg weight, yolk weight, number of eggs laid and all possible interactions. Only variables that contributed significantly ($\alpha \leq 0.05$) to the model were maintained. These variables' *p*-values are presented in the text. *Post hoc* analyses were performed for sub-samples using the same test.

Statistical analyses of female body weight, number of eggs laid by a hen and the sex ratio in relation to the social status of the hen were performed using Linear Regression and Multiple Logistic Regression (in case of sex ratios) (STATISTIX 7, Analytical software 2000).

3. RESULTS

(a) Social hierarchy

Within a cage, all females could be ranked according to the number of fights won. This resulted in a linear hierarchy, where the highest ranked females (rank = 1) dominated all other females and the lowest ranked females (rank = 4) were subordinate to all other females (table 1). In addition to the outcome of direct interactions, which are the main data for the determination of social dominance, all results were confirmed by the frequency with which a hen obtained food during a feeding trial (table 1).

Dominant females were heavier than subdominant females ($n = 20$ females, $r^2 = 0.33$, $p < 0.01$). The egg weight of a specific hen was positively correlated with her body weight ($p < 0.01$) and thus negatively with social status ($p < 0.05$). Since yolk weight and egg weight were positively correlated ($r^2 = 0.15$, $p < 0.001$), the same pattern exists for yolk weight (body weight: $p < 0.001$; social status: $p = 0.01$). Embryo weight was not correlated with any variable included in the model. Furthermore, neither social rank nor body weight had an effect on the number of eggs that were laid (body weight: $r^2 = 0.11$, $p = 0.15$; social status: $r^2 = 0.02$, $p = 0.58$) and neither were correlated with the sex ratio produced by a hen (body weight: $F_{1,13} = 0.29$, $p = 0.58$; social status: $F_{1,13} = 0.58$, $p = 0.54$) (for details, see table 2).

(b) Yolk androgens

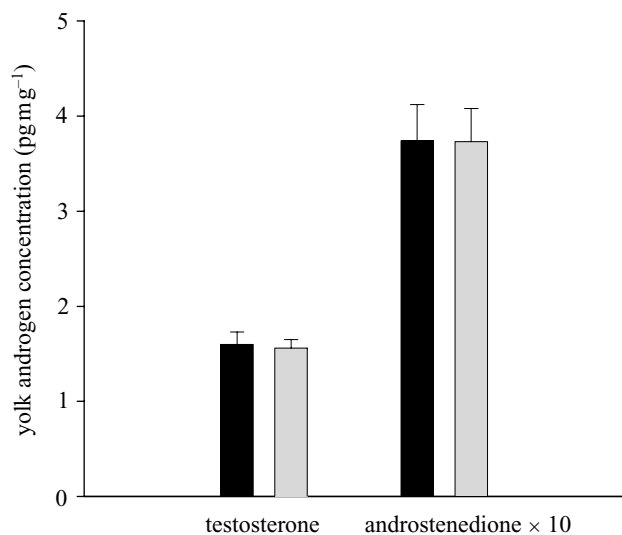
In total, 90 eggs from 14 different females were analysed for yolk hormone concentrations (for details, see table 3). When we considered the sex of the embryo as the only

Table 2. Female body weight and characteristics of her eggs in relation to her social status (mean \pm s.e.).

status	total no. of females	mean body weight	mean egg weight	mean no. of eggs	total no. of eggs	total no. of fertilized eggs	total no. females	sex ratio
1	5	1862.4 \pm 88.3	66.78 \pm 0.69	9.6 \pm 2.3	48	34	4	0.64
2	5	1731.8 \pm 71.4	67.92 \pm 0.46	10.4 \pm 1.5	52	40	4	0.48
3	5	1740.5 \pm 82.9	63.69 \pm 1.44	8.0 \pm 3.0	39	22	3	0.43
4	5	1502.6 \pm 78.4	63.49 \pm 0.57	8.6 \pm 2.2	43	24	3	0.44
sum	20				182	120	14	

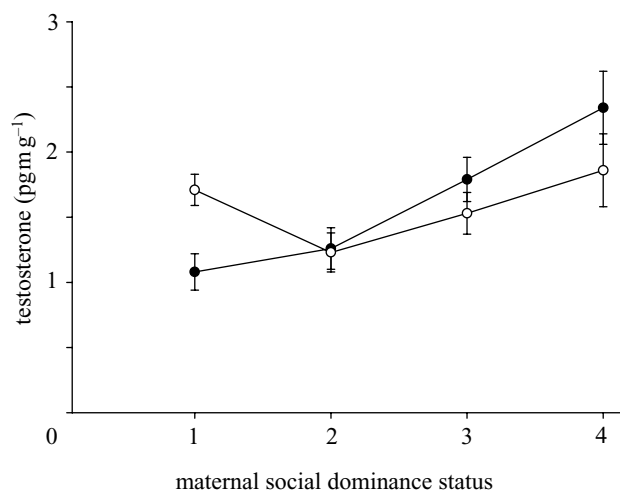
Table 3. Androgen concentrations for male and female eggs in relation to the social status of the mother (mean \pm s.e.).

status	testosterone					androstenedione			
	males		females			males		females	
	total no. of females	no. eggs	concentration	no. eggs	concentration	no. eggs	concentration	no. eggs	concentration
1	4	14	1.71 \pm 0.12	11	1.08 \pm 0.14	14	37.89 \pm 5.18	11	33.70 \pm 5.10
2	4	14	1.23 \pm 0.15	14	1.26 \pm 0.16	14	38.32 \pm 5.22	14	37.45 \pm 7.07
3	3	7	1.53 \pm 0.16	9	1.79 \pm 0.17	7	29.86 \pm 8.76	9	32.05 \pm 4.52
4	3	9	1.86 \pm 0.28	12	2.34 \pm 0.28	8	41.20 \pm 12.05	12	44.61 \pm 10.60
sum	14	44		46		43		46	

Figure 1. Yolk androgen concentrations (in pg mg⁻¹ of fresh yolk) in male (light grey bars; $n = 44$) and female (black bars; $n = 46$) eggs of White Leghorn chickens (mean \pm s.e.).

factor in the model, we found no significant difference in either yolk testosterone or androstenedione concentrations between the sexes (testosterone: $p = 0.95$, androstenedione: $p = 0.98$) (figure 1).

In respect of androstenedione, we found no relationship between yolk hormone concentrations and any variable or possible interaction included in the model. However, for testosterone we found that sex had a significant effect when the social status of the mother was included.

Figure 2. Testosterone concentrations in male (open circles; $n = 44$) and female (filled circles; $n = 46$) eggs in relation to the social dominance status of the mother (1 = dominant, 2 = 1. intermediate, 3 = 2. intermediate, 4 = subordinate) (mean \pm s.e.).

In the final model, a relationship between yolk testosterone levels and the social status of the hen ($p = 0.009$), sex of the embryo ($p = 0.002$) and the interaction of these two variables (sex \times rank: $p = 0.001$) was retained. We found that, with decreasing social position, the testosterone concentrations in the yolk of daughters increased (*post hoc* test, $p < 0.001$), but did not change in sons (*post hoc* test, $p = 0.98$). Moreover, in eggs of dominant females (rank 1), sons had significantly higher testosterone concentrations

compared with daughters (*post hoc* test, $p = 0.001$) (figure 2). In eggs of subdominant hens, female eggs had somewhat higher testosterone concentrations compared with male eggs. However, this sex effect did not reach statistical significance (rank 2–4, $p \geq 0.13$ in all cases).

4. DISCUSSION

(a) *Sex determination and yolk hormones*

This study showed that in White Leghorn chickens yolk hormone concentrations of testosterone and androstenedione did not differ significantly between male and female embryos after three days of incubation (figure 1). These results are in contrast to the data of Petrie *et al.* (2001), which showed sex-specific differences in yolk androgen levels of peafowl eggs. There may be various reasons for the discrepancies between these two studies. First, the differences in results between our study and that of Petrie *et al.* (2001) may be caused by species-specific differences in maternal hormone allocation, which is, however, unlikely since peafowl and chickens are closely related species. Second, if the sex-specific results in the peafowl are a consequence of secondary sex-specific processes during early development, a difference in incubation time (10 days in the peafowl, 3 days in this study) might explain the different results in these two studies (Elf & Fivizzani 2000; but see Eising *et al.* 2002). Third, the social position of the female, which we now demonstrate to be of significant importance, may have confounded the peafowl data.

When we included the social rank of the mother in our analysis we found a clear pattern for testosterone. With a decreasing position in the social hierarchy, testosterone concentrations increased in female eggs but did not change in male eggs. Moreover, in eggs of dominant mothers testosterone concentrations were significantly higher for male eggs than for female eggs, while this was not the case for subdominant mothers. From a mechanistic point of view, this raises the question of how females can determine the sex of the offspring when providing the yolk with hormones, which would be necessary because the sex of the embryo is determined after the yolk is formed (Sturkie 1986). Our results indicate that the androgens may be involved in sex determination, but that a simple causal link between yolk androgens and the sex of the resulting offspring (Petrie *et al.* 2001) is unlikely (figure 1). Our results indicate that if androgens (or a factor correlated with androgen levels) influence the sex of the eggs they only do so in interaction with a factor that is linked to maternal social rank (figure 2). This might be body weight (table 1) or other steroids. The latter suggestion fits with the data of two other studies. Petrie *et al.* (2001) found that testosterone and androstenedione concentrations are higher, while dihydro-testosterone and oestradiol concentrations are lower in male than in female eggs. Bowden *et al.* (2000) found a similar result for turtles, although not at the level of the individual egg but at that of the whole clutch. Stress hormones might be a relevant factor in this context since they have been shown to vary with social dominance and also to interact with reproduction (see the review in Creel 2001).

(b) *Functional aspects of maternal hormone allocation*

The sex-specific testosterone allocation fits the expectations of the sex-allocation theory (see § 1). The family *Phasianidae*, which includes the ancestor of domesticated chickens as well as peafowl, typically shows a polygynous mating system and a sexual-size dimorphism with males larger than females (Glutz von Blotzheim 1973). In these polygynous mating systems male mating success is more variable than female mating success (e.g. Guhl & Warren 1946; Graves *et al.* 1985). In addition, the larger sex is more expensive to rear due to higher food requirements (see the review in Anderson *et al.* 1993). Therefore, for parents with high rearing capacities, increased investment in sons will have a greater impact on parental reproductive success than increased investment in daughters (Trivers & Willard 1973). Thus, parents capable of high levels of investment should either shift the sex ratio of their brood towards males or otherwise intensify the resource allocation to males. We did not find a shifted sex ratio in relation to the social dominance, which is in line with an earlier study on chickens (Leonard & Weatherhead 1996). However, we found enhanced testosterone levels in male eggs relative to female eggs of dominant mothers. As yolk androgens have been shown to increase competitiveness and growth (Schwabl 1996b; Eising *et al.* 2001), they provide a mechanism for adaptive maternal investment. Thus, dominant females selectively allocate more to male offspring. Thereby they create a competitive asynchrony within their brood with an advantage for male chicks. This maternal favouritism probably enhances nutritional condition for sons, which has been shown to be of importance for males in the context of sexual selection (Gustafsson *et al.* 1995; De Kogel & Prijs 1996; David *et al.* 2000; Ohlsson *et al.* 2002). Nutrition early in the nestling phase, where maternal androgens are most likely to act, has a significant impact on the expression of sexual ornaments at adulthood and therefore reproductive success in a related species (the ring-necked pheasant, *Phasianus colchicus*; Grahn & von Schantz 1994; Ohlsson *et al.* 2002).

Low-ranking females are probably restricted in the quantity of resources that they can allocate to their offspring. In our experiment, the social status of a hen was negatively correlated with her body weight and mean egg weight, in line with earlier findings (Collias 1943; Leonard & Weatherhead 1996), suggesting a reduction in her maternal capacities. Low-ranking females are apparently restricted in the amount of resources that they obtain and can thus allocate to the eggs. Following the findings that maternal androgens have a beneficial effect on offspring growth (Schwabl 1993; Eising *et al.* 2001), subordinate females might try to compensate for lower egg quality with an increasing amount of androgens in the yolk, balancing the benefits against potential costs of testosterone (e.g. Sockman & Schwabl 2000). In line with sex-allocation theory, they allocate more yolk androgens to the offspring with the lower variance in reproductive success, favouring daughters over sons.

In conclusion, this study clearly shows that it is very unlikely that androgen concentrations alone are the decisive factor in the sex-determining mechanism. However, differential androgen deposition does take place in relation to both the sex of the egg and social rank of the mother,

and this might provide a mechanism for adaptive maternal investment.

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