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Consequences of prenatal androgen exposure for the reproductive performance of female pheasants (Phasianus colchicus)

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Electronic supplementary material

Egg injection procedure

The procedure has also been reported in Romano *et al.* (2005) and Rubolini *et al.* (2006). Briefly, the eggs were randomly assorted in two batches of 400 eggs each. Eggs of one batch were injected with T, whereas the others were injected with the vehicle (sesame oil), to serve as controls. Injections were made on the acute pole by means of a 250 μl Hamilton syringe mounting a 25-gauge, 16 mm-long needle, and the hole sealed immediately afterwards. We verified that injections effectively occurred within the yolk in preliminary trials (see Rubolini *et al.* 2006). T-eggs were injected with 40 ng T (4-androsten-17β-ol-3-one; Sigma, Germany) dissolved in 20 μl sterile sesame oil, corresponding to 2 s.d. of the total amount of T estimated for the average yolk of this species. This calculation was based on T assays performed on a sample of 73 eggs (data kindly provided by F. Dessì-Fulgheri, Università di Firenze; see Romano *et al.* 2005; Rubolini *et al.* 2006). In addition, we verified the doses injected by determining the concentration of T in a random sample of 13 eggs belonging to the same set of eggs that were injected. Mean T concentration (see below for assay) was 6.95 ng/g ± 2.28 s.d.), yielding an estimated mean amount of 73.4 ng ± 24.0 s.d.) for an average yolk of 10.6 g. Control eggs were injected with 20 μl sterile sesame oil.

Incubation, caging and rearing conditions

Injected eggs were artificially incubated, and hatching success was 43.6 %, with no differences according to treatment groups (p = 0.61, see Rubolini *et al.* 2006). Egg failures in artificially incubated eggs of the same captive pheasant population normally account for approximately 15-20%. Our experimental procedure therefore caused an increase in hatching failures by 35-40%. Chicks were individually tagged at hatching and maintained in a single large indoor aviary until day 35, when they were transferred to a large outdoor aviary. At day 210 (7 months of age), females were separated from males and 114 of them (59 from T-injected eggs and 55 from control eggs)

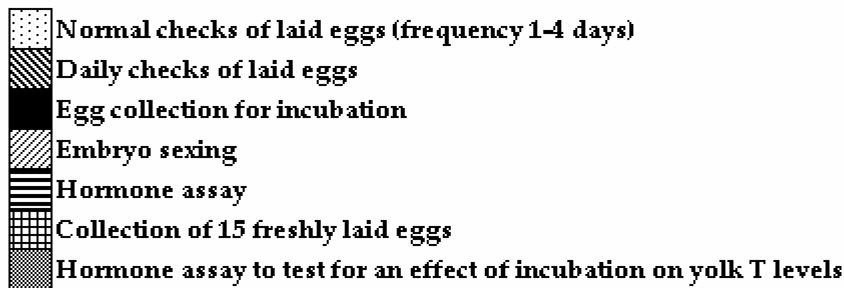
were randomly assigned to 15 indoor cages (1 x 1.15 m, height 42 cm, with sloped floors and egg catchers) in order to establish polygynous groups. Birds were reared under natural photoperiod and maintained following standard farming practices. Collected eggs laid by these females were maintained at 16°C and constant humidity for a maximum of 10 days until they were incubated in large professional incubators for 5 days to allow for embryo development (n = 568 eggs).

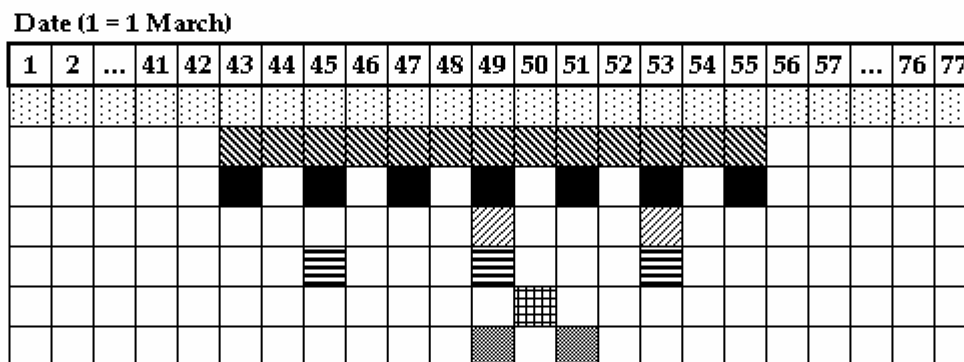
Statistical analyses

Female survival was monitored daily, so the number of females was expressed as the average number of females over the days elapsed since the last monitoring. In order to reduce the effects of random day-to-day variation in the number of eggs laid within individual cages, and of the variable monitoring intervals, data were pooled over periods of 3 or 4 days, which were regarded as cage replicates.

Egg laying rate, which varied between 0 and 1 (maximum of 1 egg per female per day), fertility and offspring sex-ratios were analyzed in binomial models. Parameters were estimated by second-order penalized quasi-likelihood estimation and their significance was tested by the Wald statistic (Goldstein 1995). We allowed for under- or overdispersion by assuming an extrabinomial error distribution (Goldstein 1995). Egg mass and composition variables were analyzed by normal error models, and significance of parameter estimates was tested using the increase in deviance (δ deviance, which follows a χ^2 distribution, with d.f. = 1) when a factor was removed from a model.

Schematic diagram of the egg sampling scheme


Normal checks of laid eggs (frequency 1-4 days)
Daily checks of laid eggs
Egg collection for incubation
Embryo sexing
Hormone assay
Collection of 15 freshly laid eggs
Hormone assay to test for an effect of incubation on yolk T levels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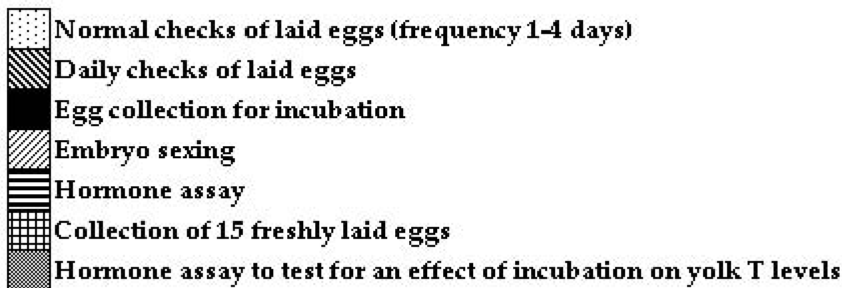
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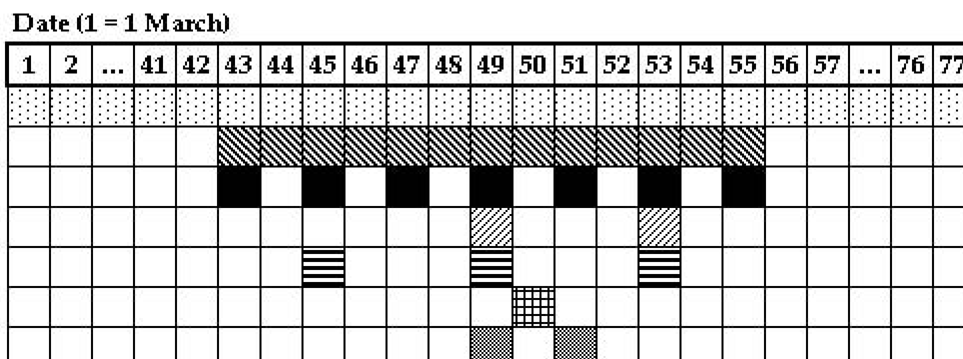
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1 2 ... 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 ... 76 77

Hormone assay and validation

The baseline yolk T concentration of the injected eggs was estimated based on 13 freshly laid reference eggs (see above and Methods), according to the protocols described in Romano *et al.* (2005) and Rubolini *et al.* (2006). Briefly, yolk T was separated from the other ether soluble components on diatomaceous earth chromatography columns, and assayed using a commercial RIA kit (ORION Diagnostica, Espoo, Finland). Based on these analyses, the dose of T injected increased the concentration of the hormone by 1.67 s.d. of the mean recorded in the 13 reference eggs (see above and Romano *et al.* 2005, Rubolini *et al.* 2006).

The hormone concentration of eggs laid by experimental females was assayed as follows. Whole yolks were carefully homogenized, and and a weighed aliquot of ca. 100 mg was diluted 1:1 with distilled water for hormone assay. To assess extraction efficiency, 20 µl labeled T were added to 200 mg of the yolk-water homogenate, which was then incubated at 37°C for 15 min to allow for equilibration of the added label with internal T. The homogenate was extracted 2 times with 4 ml 70:30 DEE:PE, the separated etherphase was evaporated under a nitrogen flow at 35 °C, redissolved in 2 ml 70 % methanol and kept at –80°C for 24 h to precipitate lipids. Samples were centrifuged at 2000 rpm and 4°C, the supernatant removed, dried at 50°C under a nitrogen flow and redissolved in PBS buffer. 50 µl of this sample were used to measure the amount of label recovered and 50 µl were assayed for T using a commercial RIA kit from DSL Inc., Texas (DSL-4000). The assay has 100% cross-reactivity with T and low cross-reactivity with other steroids (< 6%). Recoveries averaged 81%. Intra- and interassay coefficients of variation were 4.9% and 6.1%, respectively. The validity of the extraction procedure and assay for specific measurement of T was further assessed by separating T from potentially crossreacting steroids for 15 extracted yolk samples (one per cage) using Celite chromatography according to published procedures (Schwabl 1993). The correlation between T concentration as obtained using the assay with or without column separation was very high ($r = 0.91$). In addition, we assayed A4 in this subsample, using a commercial RIA kit from

DSL Inc. (DSL-4200). Yolk A4 concentrations estimated using the two methods were highly correlated ($r = 0.96$), and A4 concentration was highly correlated with that of T (assay without column separation: $r = 0.93$; assay with column separation: $r = 0.84$). The mean A4 concentration, as estimated using column separation, was 57.37 ng/g ± 20.24 s.d. ($n = 15$).

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