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Examining a pathway for hormone mediated maternal effects – Yolk testosterone affects androgen receptor expression and endogenous testosterone production in young chicks (*Gallus gallus domesticus*)

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**Abstract**

In vertebrates maternal androgens can substantially influence developing offspring, inducing both short and long term changes in physiology and behavior, including androgen sensitive traits. However, how the effects of maternal hormones are mediated remains unknown. Two possible pathways are that maternal androgens affect parts of the hypothalamus–pituitary–gonadal (HPG) axis or the sensitivity to androgens by affecting androgen receptor (AR) densities within the brain. To investigate both pathways, testosterone within the physiological range or vehicle only was injected into the egg yolk of unincubated chicken eggs and AR mRNA expression in different brain nuclei as well as plasma testosterone levels were measured in two week old male and female chicks that had hatched from these eggs. Our results showed a significant sex difference in plasma testosterone levels with males showing higher levels than females. Furthermore, AR mRNA expression as well as plasma testosterone levels were significantly lower in chicks hatched from testosterone treated eggs. These results suggest a compensatory mechanism for avoiding potential detrimental effects of high testosterone levels.

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**1. Introduction**

Embryonic development as well as offspring morphology and behavior are substantially affected by maternal effects [33]. One pathway for maternal effects is via maternal hormones, to which offspring are exposed during embryonic development. This has been extensively studied in oviparous species since their embryos develop in a sealed environment outside the mothers' body, facilitating measurements and manipulation of prenatal hormone exposure. Eggs of all avian species studied so far contain maternal steroids [48]. Furthermore, hormone deposition varies systematically between species, but also between females of the same species or between and within clutches of the same mother (for reviews see [16,22,53]). These differences are assumed to be adaptive by shaping the offspring for its future environment [22,24]. Elevation of yolk androgen levels before incubation affect offspring morphology, begging behavior, competitiveness, growth, immune function, metabolic rate, hatching time and survival (for reviews see [16,17,22,39,53]).

Despite the extensive research on hormone mediated effects, the underlying mechanism remains unknown. One possibility is an alteration of certain neural structures by affecting the organization or size of certain brain areas that are involved in the regulation of behavior. Lateralization of brain and behavior, as well as sexual differentiation, both affecting a wide array of behaviors, are affected by maternal or prenatal hormone exposure (for reviews see [3,42]).

A second, not mutually exclusive, possibility is an organizational effect of maternal androgens on parts of the hypothalamus–pituitary–gonadal (HPG) axis, which would affect endogenous hormone production. Only a few studies so far investigated this. Daisley et al. [11] treated eggs of Japanese quail with testosterone before incubation. The treatment induced behavioral changes in young chicks, but no significant difference was found in fecal testosterone levels three weeks after hatching. Muller et al. [35] reported increased plasma testosterone levels in starling chicks (shortly before fledging) hatched from eggs injected before incubation with testosterone and androstenedione in comparison to control chicks.

A third possible pathway is a change in the sensitivity to androgens by an alteration of the amounts of androgen receptors (AR) in the brain or periphery. To our knowledge, there is no study so far quantifying AR mRNA expression in birds in relation to an elevation of maternal testosterone exposure, but in vitro studies in human tissue and studies in mammals and birds showed that such autoregulatory processes exist, seem to be tissue specific and can be positive as well as negative [28,45,51] for birds see...
[37], but also [14]. The study by Toyooka et al. [51] showed an increase in nuclear AR mRNA expression in the hypothalamus of female guinea pig fetuses already 2 hours after elevating androgen levels in mothers. This effect lasted at least up to five days after the treatment, whereas no effect was found in males. Quarmby et al. [45] studied the effect of testosterone administration in castrated rats and showed that AR mRNA levels were increased after castration in brain tissues, whereas the testosterone treatment almost significantly lowered the AR mRNA expression to levels found in intact males. No females were tested in this study. Nastiuk and Clayton [37] increased testosterone levels in adult, ovariectomized canaries, inducing a significant decrease in AR mRNA expression in the HVC and forebrain after 45 minutes and 6 hours, respectively. Fusani et al. [14] however found no difference in AR mRNA expression between different seasons (breeding and non-breeding season) which are typically accompanied by either high or very low amounts of testosterone production. Clearly, changing androgen levels in the circulation can affect androgen receptor densities in the brain, but the direction of this change seems not consistent.

The aim of this study is to investigate whether maternal testosterone can affect endogenous testosterone production and/or the sensitivity to androgens in young domestic chicks. The domestic chicken is an excellent study species for this research because their endocrine system has been extensively studied, enabling reliable measurements of plasma testosterone and AR mRNA expression in the brain [15,20,32].

We injected uncumubated eggs with testosterone, elevating yolk testosterone (yolk T) levels within the natural range of maternal testosterone in the eggs of this strain, or vehicle. Eggs were subsequently incubated, and two weeks after hatching, animals were sacrificed after 2 weeks post-hatching. Animals were sacrificed after 2 weeks post-hatching. We expected our treatment to induce higher levels of plasma testosterone, and/or higher levels of AR mRNA expression. This prediction is based on previous findings that yolk T increased competitiveness in this species [43], and aggression or begging behavior in other bird species [12,18,23,36,41,50] which may be under the control of postnatal testosterone. However, the prediction is weakened by the contradictory finding in the literature on early postnatal androgen levels and AR mRNA expression as mentioned above. We quantitatively studied AR mRNA expression in the preoptic area (including different nuclei in the medial and lateral preoptic area), the BnST (bed nuclei of stria terminalis), the ventral pallidum, and the lateral septum. Previous research has shown that these nuclei contain androgen receptors and are involved in social, aggressive and copulatory behaviors [4,10,15,19,46,47] and might therefore represent nuclei that are possibly involved in early chick behavior.

2. Material and methods

2.1. Animals and housing

Freshly laid chicken eggs (LSL white Leghorn – Gallus gallus domesticus) were purchased from a commercial breeding farm and incubated at the Zoological Laboratory (University Groningen, Biological Center, Haren, The Netherlands). After hatching, 40 chicks (n = 8 per group: 2 individuals of each sex and treatment group) with ad lib food (chicken starter crumbs, Hedicr NL) and water, under 12:12 LD cycle in 78 ± 8 cm sized indoor cages with a height of 97 cm. Before treating eggs with testosterone, endogenous testosterone levels of 12 other egg yolks of the same batch were measured (for details see [43]) to calibrate the testosterone dose injected. Animals were sacrificed after 2 weeks post-hatching and brains were removed and blood samples were taken.

2.2. Hormone treatment

Before incubation was started, half of the eggs were injected with 75 ng testosterone dissolved in 0.1 ml vehicle (filtered and autoclaved sesame oil) whereas the remaining eggs were injected with vehicle only. This dose was based on the variation of testosterone levels among 12 eggs of the same batch. Total amount of testosterone for the whole yolk was 134.7 ng with standard deviation of 38.5 ng. The 75 ng resembles approximately two times the standard deviation found in the previously measured eggs. This reflects a dosage often used in such studies (see for example [8,31]. Eggs were injected by inserting a 19 g needle from the side halfway the length of the egg into the yolk; thereafter the opening was sealed with wax (for details, see [43]).

2.3. Hormone analysis

Blood samples were obtained immediately after decapitation of the animals and centrifuged. The plasma samples were stored at −80 °C until further analyses. In total we obtained sufficient volume in 31 plasma samples to measure plasma testosterone levels (ncontrol males = 7, ntestosterone males = 8, ncontrol females = 8, ntestosterone females = 8). Plasma samples and six pool samples were weighed and 50 µl radio-actively labeled testosterone (Perkin Elmer Life and Analytical Science BV) was added to enable controlling for losses during the extraction process (recovery). After an incubation time of 1 h, 2.5 ml diethyl ether/petroleum benzine (70:30) was added and samples were vortexed and centrifuged. Samples were snap frozen by a mixture of Ethanol and dry ice and decanted. The supernatant was dried under streaming nitrogen, the remaining pellet was again dissolved in 1 ml 70% Methanol and samples were stored over night at −20 °C. In the morning, samples were centrifuged, the Methanol phase was decanted and the samples dried again under streaming nitrogen. The pellet was re-suspended in 200 µl PBS buffer. Thirty microlitre of this mixture was used for measuring recoveries (average recovery rate for testosterone: 75.70 ± 1.03%).

Testosterone levels in plasma samples and pool samples were measured with one radioimmunoassay (RIA) using a commercial kit (Active Testosterone Coated-Tube RIA DSL-4000 kit, Diagnostic Systems Laboratories) with a sensitivity of 0.08 ng/ml testosterone and cross-reactivities of 5.8% with DHT (5α-dihydrotestosterone) and 2.3% with A4 (4-Androstene-3,17-dione). The dilution curve ran parallel to the standard curve (percentage binding of the standard curve between 20.19% and 91.16%). The chick plasma samples had an average percentage binding of 75.97 ± 1.51%. The intra-assay variation was on average 5.61 ± 1.52%.

2.4. AR mRNA expression

After sacrificing the animals, brains were immediately removed, frozen on liquid nitrogen (−80 °C) and later transported on dry ice to the Max-Planck-Institute for Ornithology (Seewiesen, Germany), where they were sectioned coronally (from anterior to posterior) at 20 µm. Cryostate temperature was kept stable at −17 ± 2 °C. Brain slices were mounted on microscope slides (Fisher Superfrost Plus) in consecutive series of five and remained frozen (−80 °C) until further analysis.

Brain slices of 26 chicks could be used for the quantification of AR mRNA expression (ncontrol males = 7, ntestosterone males = 6, ncontrol females = 7, ntestosterone females = 6). Every fifth microscope slide was incubated for 40 seconds in 0.1% Thionin (Serva, Heidelberg, Germany) for Nissl staining to enable identification of brain areas according to the chicken brain map [44]. Based on the location of the anterior commissure, sections for the in situ hybridization were selected enabling the best possible comparison between treatment...
groups and sexes. This brain level (from 500 μm rostral to 500 μm caudal to the anterior commissure) allows the analysis of the preoptic area, the bed nucleus of the stria terminalis (BnST), the ventral pallidum and different septal nuclei (Fig. 1A). For the quantitative comparison of AR mRNA expression, the mRNA expression was summed over all nuclei. The amygdala that is visible at this
Sex differences is redundant due to the standardization, testing a and treatment and their interaction as factors. Although testing distributed; all the standardized residuals of the model, both being normally distributed; all data and for the standardized residuals of the model, both being therefore LOG transformed, resulting in a normal distribution.

The in situ hybridization procedure is described in detail in Metzendorf et al. [32], which is a modification of the method by Whitfield et al. [55]. Riboprobes, using chicken AR cDNA as a template, were radioactively labeled with 35S-CTP. The chicken AR was kindly provided by Dr. Yamada (see also [29]). The radioactive signal was detected by autoradiography using Kodak Biomax MR films. After an exposure period of 14 days, the films were developed with Kodak LXP24 developer using Kodak AL4 for fixation and scanned for further analyses (at resolution of 300 dpi). Afterwards, slides were dipped in photo-emulsion (Kodak NTB2) for high resolution autoradiography allowing verification of the positive AR expression and the identity of the brains areas selected for the densitometry. These autoradiograms were exposed for 2 weeks and developed with Kodak LXP24 developer.

The quantification of AR mRNA expression was based on the autoradiographic films using the program Image J (1.40 g; Wayne Rasband, National Institutes of Health, USA). The autoradiographic films darken where being exposed to the radioactive signal used in the in situ hybridizations. The resulting differences in grey intensities (darker grey intensity in areas containing AR mRNA and lighter grey intensity in regions without AR mRNA expression) are used for quantifying AR mRNA expression. To standardize the quantification process, the grey intensity of the background (areas without AR expression) was measured for all microscope slides that were used. A standard value was calculated based on all slides that were used and was subtracted from the background, marking only areas that were considerably darker than the background. The area including the nuclei of interest is then selected (see Fig. 1B) and the amount of pixels contained in the marked area is counted and compared between treatment groups.

Females were analyzed in a different batch than males in which autoradiography films were considerably darker. We therefore had to select a different threshold than for the males. The resulting sex difference in the total amount of marked pixels counted was considerable (males: 578.61 ± 119.75 pixels; females: 260.45 ± 58.86 pixels) and although this might be biologically relevant, in this study it is obscured by the differences in the in situ hybridizations and autoradiographic films and we therefore do not discuss this any further in this paper.

2.5. Statistical analysis

Plasma testosterone levels were not normally distributed and therefore LOG transformed, resulting in a normal distribution (Kolmogorov-Smirnov Test was applied for the LOG transformed data and for the standardized residuals of the model, both being normally distributed; all p values >0.15). Treatment and sex effects were tested in a two-way ANOVA using SPSS (16.0 for Windows).

The analysis of AR mRNA expression was first corrected for sex differences which might rather be due to artefacts from different in situ hybridizations than actual biological differences (see above). This correction entailed subtracting the average of the male or female population from the individual male or female count, respectively. This procedure standardizes the average of males and females to zero and diminishes differences in the total amount of pixels counted and allowed testing the treatment effect independently of sex.

The AR mRNA expression data were normally distributed (Kolmogorov–Smirnov Test was conducted for the data itself and the standardized residuals of the model, both being normally distributed; all p values >0.10); hence a two-way ANOVA using sex and treatment and their interaction as factors. Although testing sex differences is redundant due to the standardization, testing a possible interaction effect is still important, since the treatment effects might be different for the two sexes.

For post hoc comparisons t-tests were used. We controlled for multiple testing by adjusting the critical α-levels according to Hochberg’s step-up procedure ([26], see also [27]).

3. Results

3.1. Plasma testosterone

The elevation of yolk T decreased plasma T in young chicks (Fig. 2). The ANOVA showed a non significant interaction effect (dfm=15, dfb=16, dfc=15, dTe=2.046, p=0.164) whereupon the interaction effect was removed from the model, allowing to test for main effects. The remaining treatment effect was significant (F2,28 = 4.804; p = 0.037) as well as the difference between the sexes (F2,28 = 14.039; p = 0.001), with males showing, as expected, higher levels than females.

Although the interaction effect was clearly non-significant, we performed post-hoc tests to explore the treatment effect in more detail, but the results should be taken with caution. These tests showed a significant treatment effect within males, but not within females (males: t-test for equal variances: n = 15, df = 13, t = 2.743, p = 0.017 (critical α-level: p = 0.025); females: t-test for equal variances: n = 16, df = 14, t = 0.581, p = 0.570 (critical α-level: p = 0.05)). Within the control group, males and females differed significantly, whereas the sex difference within testosterone animals was borderline significant (control: t-test for equal variances: n = 15, df = 13, t = -3.519, p = 0.004 (critical α-level: p = 0.025); testosterone: t-test for equal variances: n = 16, df = 14, t = -1.832, p = 0.088 (critical α-level: p = 0.05)).

3.2. AR distribution and densities

Areas in which AR mRNA expression was present are indicated in Fig. 1A. Considerable expression was found in the purkinje cell layer of the cerebellum (Fig. 3A), the lateral septum, the bed nucleus of the stria terminalis (Fig. 3B), throughout the amygdala (Fig. 3C), the preoptic area including the medial preoptic nucleus, the mediobasal hypothalamus (Fig. 3E), and the periaqueductal gray and the nucleus intercollicularis of the midbrain. That darkened areas seen on the autoradiography films were due to AR mRNA expression was validated by photomicrographs from the dipped slides (Fig. 3).

Fig. 2. Plasma testosterone levels (average ± SEM in ng/ml) in 2 weeks old male and in female chicks; white squares indicate control groups, filled squares chicks from testosterone injected eggs.
In males and females testosterone reduced AR mRNA expression (Fig. 4). The interaction effect between sex and treatment was not significant (ANOVA; \( n_{\text{males}} = 13, n_{\text{females}} = 13, n_{\text{control}} = 14, n_{\text{testosterone}} = 12; F_{3,22} = 0.738, p = 0.400 \) (Fig. 4)). To test an overall treatment effect, the interaction and sex were removed from the model. The remaining treatment effect showed a significant decrease in AR mRNA expression in the measured nuclei (\( F_{1,24} = 4.380, p = 0.047 \)).

As for the T level data, we performed a post-hoc test to explore treatment effects within the sexes, showing no significant effects (t-test for unequal variances; males: \( n = 13, df = 7.278, t = 1.742, p = 0.123 \) (critical \( \alpha \)-level: \( p = 0.025 \)); females: \( n = 13, df = 7.020, t = 1.394, p = 0.206 \) (critical \( \alpha \)-level: \( p = 0.05 \)). The lack of treatment effect in the post hoc comparison might be partly explained by the small sample sizes, at least in males in which the treatment effect seems to be stronger than in females (see Fig. 4).

4. Discussion

Despite extensive research on the effects of maternally transferred androgens on behavior and morphology of offspring, very little is known about the underlying mechanisms mediating these effects. The aim of the present study was to test two possible pathways; a change in plasma testosterone levels in young chicks and a
change in the sensitivity towards androgens within the brain indicated by a change in AR mRNA expression in the relevant brain areas.

In general the expression pattern of the AR mRNA in the chicken brain was as expected from previous work that mapped the testosterone binding [7,30] or the androgen receptor protein [49] in the chicken. Further, the overall distribution of AR mRNA in the chicken brain was similar to the distribution of AR protein in the closely related Japanese quail [6,52] and similar to the distribution of AR mRNA of songbirds, with exception of areas present in the forebrain of songbirds [32]. Even brain regions that are rarely reported as expressing AR mRNA such as the cerebellum [32] were detected (see also Fig. 2), suggesting that the application of the in-situ hybridization protocol to the chicken brain delivered sensitive results. A detailed documentation of the AR mRNA distribution in the chicken brain shall be published elsewhere. Our study showed a suppressive effect of elevated yolk T on both testosterone production and AR mRNA expression.

Yolk steroid hormones decrease in the course of embryonic development [13] and previous studies have shown that yolk steroids are taken up by the embryo [40,54]. Maternally derived hormones in the yolk are still available to the embryo at the time that the gonads start to produce endogenous hormone production (around day 6) as well as at the time, the HPG axis is activated (day 11.5 and 14.5) [40,54,56,57]. This overlap in time between endogenously produced hormones, the activation of the HPG axis and the availability and consumption of maternal hormones makes it possible for maternal hormones to affect the endocrine system of the growing offspring. Our data support such an effect, given that testosterone levels and androgen receptors are affected by our treatment. The decrease in expression of brain androgen receptor mRNA shows that yolk hormones of maternal origin can affect the brain. However, it seems unlikely, that the effect on testosterone one plasma levels was due to such an effect. Lower AR mRNA expression in the hypothalamus would decrease the negative feedback from circulating androgen levels on the gonadal production of androgens, which would result in higher levels of T in the experimental chicks and not in lower T levels as we found them. Therefore the lower T levels in the experimental chicks and the decreased AR mRNA expression suggest the gonads as a putative target for the effects of elevated maternal testosterone on testosterone production.

Lower testosterone production could then be responsible for lower androgen receptor mRNA expression since one possible mechanism how endogenously produced testosterone could affect AR mRNA expression is via autoregulation. Circulating levels of androgens can affect androgen receptor genes and positive as well as negative autoregulation has been shown (see introduction, for a review see [28]). Our treatment induced a decrease of endogenously produced testosterone in young chicks and assuming that AR mRNA expression is affected by autoregulatory processes, this decreased T production lowered the AR mRNA expression in the measured nuclei. An alternative possibility could be that not autoregulatory processes are responsible for the lower AR mRNA distribution, but that yolk T levels affect endogenous testosterone production and AR mRNA distribution separately. This would imply though, that high yolk levels permanently down-regulate AR mRNA expression via molecular imprinting. But this seems less likely given that AR mRNA expression is dynamic and rather dependent on acute testosterone levels than on yolk T levels. Another possibility would be that the nuclei containing AR mRNA expression change in size or number due to the treatment. As indicated in Fig. 1B, we only quantified the whole AR mRNA expression in the measured area, making it impossible to separate the different nuclei in our analysis. Nevertheless, our treatment significantly affected the expression of AR mRNA in this area and follow up experiments would include a detailed analysis of the separate nuclei showing the highest levels of expression (see Figs. 1A and 3).

Previously published articles showed an increase in plasma testosterone levels in chicks hatched from androgen treated eggs compared to control chicks [35] or were not able to show a difference between testosterone and control chicks [11]. The latter study used fecal samples collected in social isolation to measure circulating testosterone levels which are assumed to reflect plasma levels of approximately 30 minutes before excretion. But short-term stress such as induced by social isolation has been shown to affect T secretion at least in the chicken [25] which could possibly mask treatment effects (see also [21]). In addition, the effects of yolk T on the endocrine system may be species specific, as is, for example, the effect of yolk T on hatching time [53]. This species specificity may be related to species specific life-histories, differentially affecting costs and benefits of elevated exposure to maternal androgens. For example, the potential benefits of yolk T on aggression and begging, established for altricial (as used in [35] and semiprecocial species [22,17,53], might be less relevant for chickens where the chicks do not beg for food and do not show overt aggression towards each other. The potential costs may also differ. One such cost, immune suppression [1,9,34,38] for reviews see [16,17,22,53], may be much more relevant for precocial species like the chicken, where chicks move freely among conspecifics, enhancing the risk on infections compared to altricial young that stay in their nests. This may explain why in the domestic chick elevated levels of yolk T down regulate androgen exposure after hatching in order to avoid the previously mentioned potential costs on immune function and aggression. In addition, yolk T also seems beneficial for young chicks in competitive situations that do not necessarily involve aggression. Interestingly, we have shown in the same chicks used for this study that yolk T elevates (success in) food competition in the young chick, while this behavior at the time it is performed is independent from testosterone [43].

Another, not yet discussed possibility how effects of maternal testosterone might be mediated is via aromatase activity. Aromatase is the enzyme converting testosterone into estradiol which is involved in the expression of social and sexual behaviors (for a review see [2]). Aromatase activity can be regulated via different pathways, with steroid hormones being one of them (for a review see [5]). To further disentangle the mechanisms by which maternal androgens affect offspring behavior it would be important to combine our measurements with measuring aromatase activity and estradiol (E2) levels, as well as circulating levels of dihydrotestosterone (DHT), a metabolite of testosterone with a high binding affinity to the androgen receptor. However, since circulating levels of DHT and E2 are generally much lower than that of T, it is highly unlikely that the lower levels of T in the experimental birds were due to higher conversion rate of T to DHT and or to E2.

Our results so far suggest that the effects of maternal testosterone are mediated via autoregulatory processes. The decreased plasma testosterone levels seem to cause a decrease of AR mRNA expression in the measured brain nuclei. It remains elusive whether these differences are still present in adult animals and thereby affecting reproductive success or whether this effect is adaptive in young animals to avoid possible detrimental effects of high plasma testosterone levels.

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